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RECENT CHEMICAL INVESTIGATIONS OF BACTERIAL TOXINS

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The chemical investigation of bacterial toxins holds much of practical and theoretical significance not only in immunology but also in physiology and chemistry. The ultimate objectives of

these studies are to determine the mode of formation of toxins, their nature, and relationships between their chemical and biological properties. This review will indicate some of the paths by which these objectives have been approached.

True toxins such as those of diphtheria and tetanus may be distinguished from other bacterial poisons by high antigenicity and the production of well-defined physiologic effects when minute amounts are injected into susceptible animals. Although the term toxin has been used to denote any poison of unknown nature, it is used in this paper to mean an antigenic poison. It is difficult to draw the line between "exotoxins" and "endotoxins" because antigenic poisons possess all degrees of toxicity and antigenicity. There are also marked differences in the ease with which toxins are liberated from bacterial cells.

Well-known biologic properties of toxins and immunological phenomena common to toxins and non-poisonous antigens will be discussed in this review only when related to the nature or mode of action of toxins. The reaction between toxin and antitoxin could be discussed in detail more profitably in a review of antigens and antibodies in general, because this reaction probably does not differ essentially from the reaction between any other antigen and the corresponding antibody.

Nothing at all will be said about toxins such as that from *Clostridium welchii*, the toxic substances derived from gram-negative cocci, or the fibrinolysins. In general, what chemical work has been done on these is similar to that on other toxins described in this review.

PART I. THE PRODUCTION OF BACTERIAL TOXINS

1. The use of media of simplified composition

Progress in studies on the mode of formation and the nature of toxins will be greatly facilitated when toxins are produced on media of known composition, or, at least, on media much simpler than those at present in use.

Mueller (1922 to 1937) has worked out the cultural requirements of the diphtheria bacillus so that abundant growth of some toxigenic strains may be obtained in a medium consisting of

simple organic and inorganic substances. Pappenheimer and Johnson (1936), in extending the observations of Locke and Main (1931), Scheff and Scheff (1934), and others, have shown that definite low concentrations of iron and copper are essential to toxin production. Previous failure to produce it in Mueller's medium was due to the inhibitory effect of an excess of iron. Pappenheimer, Mueller, and Cohen (1937) have described the production of potent diphtheria toxin in a medium in which the organic constituents are certain amino acids together with pimelic acid, beta-alanine, and nicotinic acid. This medium is not synthetic in the strict sense of the word, because some of the purified constituents were obtained from natural sources and may have contained impurities which stimulate toxin production. In this connection may be mentioned the finding of Pappenheimer and Johnson (1937) that an unknown inorganic constituent of soft glass stimulates the production of toxin in simplified media.

Media consisting of amino acids and small amounts of partially purified but unknown constituents have been developed by Knight, Fildes and their co-workers (1933, 1936) for growth of the staphylococcus and for *Clostridium botulinum*. Knight (1937) has found that the accessory substances necessary for the growth of the staphylococcus are nicotinic acid and vitamin B_1, or closely related substances. However, toxins have not yet been produced in significant amounts in these media. Burrows (1933), using an amino acid medium, was able to produce weak toxin from type A strains of *Cl. botulinum* but not from type B strains. Tryptophane seems to stimulate slightly the production of toxin. Tani (1934) obtained growth and production of toxin by *Cl. botulinum* in a biuret-negative medium made from a sulphuric acid hydrolysate of Witte peptone.

For purposes of subsequent purification of toxins, we may use protein-free media of simplified but unknown composition from which the toxin may be precipitated selectively. The Wadsworth-Wheeler medium (1934) contains no proteins precipitable by acid but does contain proteoses precipitable by ammonium sulphate, alcohol, or acetone. Diphtheria toxoid may be precipitated by acid in a relatively pure state from this medium, but

the toxin is damaged by acid. This medium might be useful for the production and purification of other toxins that remain undamaged by precipitation with acid. A similar medium has been used by Sommer (1937) for botulinus toxin. For the production of diphtheria toxin, Pappenheimer and Johnson (1937) have described a gelatin hydrolysate medium to which certain amino acids and accessory substances have been added. It contains no substance precipitable by ammonium sulphate, and diphtheria toxin produced in it may be considerably purified simply by salting out. Holt (1937) and McLean (1937) have obtained growth and toxin production by *Staphylococcus aureus* in a medium made with the dialysate from nutrient broth. This medium contains no nitrogenous substances insoluble in saturated ammonium sulphate.

2. The possible relationship of oxidation-reduction systems to the formation of toxins

Although direct proof is lacking, a number of observations seem to link the formation of toxins to the activity of respiratory enzymes and other substances concerned in oxidation-reduction processes. In 1931, Coulter and Stone described a complex porphyrin, apparently containing both copper and iron, which is present in the toxic filtrates from cultures of *Corynebacterium diphtheriae* and is not formed by non-toxigenic strains.[1] It may, however, be formed by yeast and certain other organisms. The amount of porphyrin as judged by the intensity of the absorption spectrum is parallel to the amount of toxin formed. These observations were confirmed by Wadsworth, Crowe, and Smith (1935) who also showed that the porphyrin can be adsorbed out of toxic filtrates by charcoal. Wheeler and Crowe (1936) found that removal of porphyrin from, or its addition to, cultures of *C. diphtheriae* does not affect the formation of toxin. Pappenheimer and Johnson (1937) found that the amount of toxin

[1] Since this article has gone to press another paper by C. B. Coulter and F. M. Stone (Proc. Soc. Exp. Biol. Med., 1938, **38**, 423–425) has appeared in which evidence is presented that the pigment in diphtheria culture filtrates is a zinc coproporphyrin compound.

and the amount of porphyrin can be increased or decreased in parallel by varying the concentration of iron in the medium.

These observations and the fact that iron and copper are essential for the production of diphtheria toxin indicate that the porphyrin found in the filtrates may take part in the formation of toxin in the bacillus although it has no effect when free in the culture medium.

Urban and Eaton (1937) have shown that the porphyrins derived from *C. diphtheriae* are reversibly oxidized by oxygen in the presence of reduced cytochrome C and form a redox system with lactoflavin, but, contrary to the observations of Coulter and Stone (1931), these porphyrins are not directly oxidized by potassium ferricyanide.

Levaditi and his co-workers (1934) have called attention to another substance, with an absorption band at the ultraviolet end of the spectrum, which they claim is parallel in amount to the amount of diphtheria toxin formed and is absent from culture filtrates of non-toxigenic strains. This is denied by Ottensooser, Krupski, and Almasy (1935).

Although iron, copper, and cysteine are essential for growth and toxin production by the diphtheria bacillus (and probably other organisms), excess of any one of these substances inhibits the formation of toxin. (Locke and Main, 1931, Pappenheimer and Johnson, 1936). Kligler, Liebowitz, and Berman (1937) report that ascorbic acid added to the culture medium in a concentration of 0.1 mg. per cc. markedly reduces the formation of diphtheria toxin. The reported inhibitive effects of cysteine and ascorbic acid may have been due to the introduction of an excess of iron as an impurity in these substances.

Burky (1933) observed that when toxigenic strains of *Staphylococcus aureus* are grown anaerobically no pigment and no hemotoxin are formed but the concentration of lethal toxin is the same as in filtrates from aerobic cultures. On the other hand, anaerobic cultivation of the streptococcus or pneumococcus does not reduce the production of hemotoxin. The production of diphtheria and Shiga dysentery toxins is inhibited by anaerobic cultivation of the organisms. McBroom (1937) has found a cor-

relation between the ability of strains of staphylococcus to reduce methylene blue and to form hemotoxin. Kodama (1936) has reported that a thermolabile substance extracted in parallel with the cytochrome pigments from muscle stimulates the production of erythrogenic toxin by the hemolytic streptococcus. These observations constitute the fragmentary evidence that oxidation-reduction processes in the bacterial cell may be intimately associated with the formation of at least some of the toxins.

3. Other factors affecting the formation of toxins

Because of the use of complex media made up of substances of indeterminate composition and purity, much of the work on toxin production, although of undoubted practical value, has little significance in the present connection. For this reason an extensive review of the literature on this subject has not been undertaken.

A constituent of the medium need not necessarily act directly either by stimulating growth or toxigenicity. It may act by removing an inhibitory factor, by effecting changes in pH or reducing conditions during growth, or by protecting the formed toxin from destruction. Traces of unknown impurities in an "essential" substance may stimulate the formation of toxin. The success of Taylor (1935) in producing very strong diphtheria toxin in a hog-stomach-digest medium is due to factors as yet unknown. The work of Pappenheimer, Mueller, and Cohen (1937) and Pappenheimer and Johnson (1936, 1937) indicates that in a medium containing the organic and inorganic substances required for growth of the diphtheria bacillus, the production of toxin may be brought about by the addition of the proper amounts of inorganic substances. The latter investigators have pointed out that the amount of iron necessary to inhibit toxin formation is considerably less than that found in normal tissues. An excess of iron may also occur in peptones or sugars. Most methods for preparing media for the production of diphtheria toxin involve a step in which phosphates of metal ions are precipitated in alkaline solution. The excess of iron inhibitory to toxin production is carried down and removed in this precipitate.

The manner in which carbon dioxide and soft agar act in stimulating the formation of toxin by the staphylococcus has been the subject of considerable investigation. Bigger (1933) found that good toxin is produced by some strains in a medium containing glycerol and phosphate buffer without carbon dioxide; but McLean (1937) has had less success in substituting other buffers for carbon dioxide. This suggests that the carbonic acid may act as a buffer, or maintain the pH at the optimum level for toxin production. Carbon dioxide as such, and independently of its effect on pH, stimulates the growth of bacteria, and it may in some way affect the metabolic processes concerned in the formation of toxin.

Until recently the use of soft agar for the production of staphylococcus toxin has been considered essential. However, McLean (1937) has found that after adsorption from the medium of an inhibitory substance by kieselguhr, kaolin, filter paper, or cellophane, good toxin may be produced without agar. McLean believes that agar acts in a similar way.

Jordan and Burrows (1935) report that the production of enterotoxic substances by strains of staphylococcus concerned in food poisoning is greatly increased by adding starch to the medium and cultivating the organisms on soft agar under 20 per cent carbon dioxide. After repeated transfer to this medium certain strains of *Streptococcus viridans*, *Bacillus proteus*, and members of the colon-typhoid group also acquire the ability to produce gastro-intestinal poison. It is possible that the enterotoxic substance is a metabolite, not a true toxin, because it is non-antigenic, and, unlike other well-defined toxins, it is soluble in organic solvents (Jordan and Burrows, 1933).

4. Toxins as products of secretion, autolysis, or the action of enzymes on the medium

The production of diphtheria toxin in a medium containing only substances of the degree of complexity of amino acids, and the production of other protein-like toxins in media containing no proteins or proteoses makes it unlikely that these toxins are formed by enzymic degradation of a constituent of the culture

medium. It seems rather that toxins are synthesized in the bacterial cell and then liberated, by diffusion into the medium or by disruption of the cell. Most toxins are intermediate in their properties between two extremes represented, on the one hand, by diphtheria toxin, which appears in the medium even during the first hours of growth and is easily washed out of the bacilli, and, on the other, by the toxic substances in the bodies of the colon-typhoid organisms which are liberated only by prolonged autolysis, tryptic digestion, or extraction with acids.

Nelson (1927) found that intact botulinus bacilli are very toxic and their toxicity is not appreciably diminished by washing. The toxin is apparently combined with a protein of the bacterial cell which can be removed by peptic digestion. More recently Sommer (1937) has reported that botulinus toxin having almost the same activity as the purified toxin separated by him from filtrates can be obtained by dissolution of the dried bacilli in phosphate buffer.

Both the neurotoxin and the enterotoxin of the Shiga dysentery bacillus exist in the bacterial bodies (Boivin and Mesrobeanu, 1937b). The neurotoxin is liberated from the cells by diffusion in an alkaline medium, but the enterotoxin is liberated only by autolysis. Hansen (1936) has obtained strong dysentery toxin by grinding the dried bacilli in water and concentrating the toxin by adsorption and precipitation. Gildermeister and Grillo (1935) obtained increased production of toxin by growing the cultures in broth at pH 8.8 inside a cellophane bag immersed in broth. This was attributed to the escape of metabolic products through the membrane, thus permitting better growth of the bacilli.

In 1934, Weld described a heat-labile hemotoxin of the streptococcus which, after intravenous injection, kills mice and produces extensive intravascular hemolysis. Unlike the pneumococcus hemotoxin which is generally liberated by breakdown of the cells and the staphylococcus hemotoxin which is formed as a soluble substance during growth, the streptococcus hemotoxin is best obtained by treating the organisms with serum. Several successive serum extracts contain hemotoxin of equal potency, and extraction of a larger mass of the cocci with the same amount of serum does not increase the potency. This indicates saturation

of the serum or limitation of the amount of toxin formed by the availability of something in the serum. Schluter and Schmidt (1936) have repeated the work of Weld, and have been unable to obtain hemotoxin by extraction with Tyrode solution or solutions of gelatin, gum arabic, or peptone. With solutions of serum albumin or globulin only weak toxins were obtained. Dialyzed serum failed to extract the toxin but addition of sodium chloride restored its extracting power. Dilution or concentration of the serum also diminished the extracting power. The ability of the streptococci to produce hemotoxin diminishes after 14 hours of growth. Extraction of the organisms with saline, ether, or alcohol also destroys the ability to yield hemotoxin on subsequent treatment with serum. It is possible that this hemotoxin of the streptococcus is not extracted by the serum but is formed by the action of an enzyme of the organism on some constituent of the serum.

PART II. THE PURIFICATION AND CHEMICAL NATURE OF BACTERIAL TOXINS

1. Concentration, partial purification, and separation of toxins

This section will be devoted to the applications of chemistry in the study of toxins which have been concentrated or have been separated from a mixture of toxins but have not in most cases been purified to any great degree.

Procedures such as precipitation with alcohol, acetone, or ammonium sulphate, dialysis or ultrafiltration, and evaporation at low pressure and temperature have long been used to concentrate bacterial toxins. Such methods usually do not effect a great amount of purification except where specially designed and simplified media have been used for production of the toxin. However, concentration may be useful in the study of bacterial products having poorly defined biological properties; and simple chemical procedures may sometimes be used to separate a mixture of two or toxins produced by the same organism.

In considering the destructive effects of a chemical procedure two criteria of the amount of alteration of the toxin should always be observed:

(1) The final yield of toxin (measured in units, such as skin

test dose, minimal lethal dose, or flocculating unit) in the purified or concentrated sample should be a major fraction of the total toxin in the original material.

(2) The toxicity per gram of dry weight in the final preparation should be equal to or greater than that of the original toxin.

a. Toxins of the hemolytic streptococcus, pneumococcus, and staphylococcus. Scarlatinal toxin may be concentrated by fractional precipitation with ammonium sulphate or sodium chloride according to the methods used by Huntoon (1924), Dick and Boor (1935) and others. Acetone has been used as a precipitant by Wadsworth and Quigley (1931). Precipitation with neutral salt has yielded a ten- to twenty-fold purification of the toxin without much loss of toxicity. Simple acetone precipitation has effected a five- to ten-fold purification. These methods undoubtedly involve precipitation of much inactive material from the culture medium.

In 1929 Korschun, Krestownikowa, and Rjachina precipitated scarlatinal toxin with sodium chloride and then with alcohol. The alcohol precipitate was redissolved in acidified water to separate the toxin from an insoluble nucleoprotein. The resulting toxic substance is stated to be a polysaccharide containing nitrogen. A similar substance was obtained by Kodama (1936). Huntoon, and Dick and Boor reported that their scarlatinal toxins were destroyed by tryptic digestion, but Kodama used tryptic digestion as a step in the purification of his scarlatinal toxin. Recently Stock (1937), using methods similar to those of the Japanese and the Russian investigators, has obtained a toxin of considerable activity which gives 60 per cent of reducing sugars on hydrolysis. Stock showed, however, that a similar polysaccharide can be isolated from commercial peptone. Apparently, scarlatinal toxin has not yet been isolated in a degree of purity sufficient to warrant definite conclusions as to its nature.

Separation of various substances giving skin reactions and of other toxic products from hemolytic streptococci has been partially successful but much remains to be done. Hooker (1936) has called attention to the multiplicity of toxins or poisons, elaborated by the hemolytic streptococcus, which may play a

part in infections with this organism. In 1934, Hooker and Follensby demonstrated the existence of what they termed A and B toxins of the hemolytic streptococcus. The B toxin is present in the fraction precipitated by 0.60 saturated ammonium sulphate solution, while the A toxin is precipitated at 0.75 saturation. Hooker and Follensby found strains of streptococci which produce mostly A or mostly B toxin. The NY5 strain produces A and B while the Dick strain produces only A. The two toxins differ in their stability to various physical and chemical agents. The B toxin resembles a polypeptide or protein while the A toxin in many respects departs from the properties commonly attributed to a protein. It is interesting to note that the B toxin is digested by trypsin whereas the A toxin resists digestion by pepsin, pancreatin, or trypsin.

From hemolytic streptococci and their culture filtrates, Kodama (1936) has separated several substances which give skin reactions. One of these is a scarlatinal toxin which resists tryptic digestion and which Kodama believes is similar to the A toxin of Hooker. In addition, Kodama describes an alcohol-soluble, acetone-insoluble polypeptide which gives reactions of an allergic nature in certain adults, and a nucleoprotein which also gives allergic skin reactions. The importance of separating toxic from allergic factors in streptococcic filtrates is evident in view of the discussions of these factors in papers such as those by Hooker (1933), Ando, Kurauchi, and Nishimura (1930), and Cooke (1928).

Rane and Wyman (1937a) have demonstrated a definite flocculation of scarlatinal toxin and antitoxin by using toxin concentrated and partially purified by precipitation with ammonium sulphate. O'Meara (1935) and others had previously obtained flocculation by using enormous volumes of unconcentrated toxin relative to the amount of antitoxin, and by incubating for a long time. The concentrated toxin prepared by Rane and Wyman flocculates with a suitable serum in 15 minutes. Although other investigators have failed to demonstrate a relation between the flocculation value and the skin test dose of scarlatinal toxin, Rane and Wyman report relatively constant values of about

60,000 skin test doses (as determined on rabbits) per flocculating unit, using the National Institute of Health unit of antitoxin. In most details the flocculation of scarlatinal toxin closely resembles the Ramon reaction with diphtheria toxin.[2]

The demonstration that hemolytic streptococci and pneumococci form lethal substances has been accomplished by purifying and concentrating these substances. The concentrated streptococcus toxin prepared by Korschun and his collaborators was sometimes fatal to rabbits in doses of 0.02 gram. Rane and Wyman (1937b) report that strong toxins containing 10 to 80 flocculating units per cubic centimeter kill adult rabbits in doses of 5 cc. Young rabbits are more resistant.

In addition to the toxins of hemolytic streptococci just discussed, three streptococcus hemolysins have been described, and two of these are apparently toxic for experimental animals. As was mentioned in the preceding section, the heat-labile substances extracted from streptococci with serum (Weld, 1934) kill rabbits and mice with extensive *in vivo* hemolysis. More recently Czarnetzky, Morgan, and Mudd (1938) have prepared a heat-stable hemolysin by extraction of frozen and dried ("lyophile") streptococci with ether. A crystalline derivative of this hemolysin is also hemolytic and lethal for mice and rabbits in doses of approximately 0.16 mg. per kilogram of body weight. These hemolysins and that described by Weld have not yet been shown to be antigenic. Consequently, they must be provisionally classified as poisons rather than as true toxins. Besides these two hemolysins which are oxygen-stable, Todd (1932) has described an oxygen-labile streptococcus hemolysin which is antigenic and probably similar to pneumococcus hemolysin. This hemolysin may be related to the oxygen-labile antigen of Group A streptococci described by Czarnetzky, Mudd, Pettit, and Lackman (1938).

By concentrating culture filtrates of pneumococcus type III by ultrafiltration, Coca and his associates (1937) have obtained a

[2] Ramon and his collaborators (1937a) report that tetanus anatoxin, concentrated by precipitation with trichloracetic acid, flocculates rapidly with antitoxin.

preparation which kills mice in 1 cc. doses and produces skin reactions in rabbits. Dick and Boor (1937) have obtained a similar substance by precipitation with ammonium sulphate. The fatal dose for mice is approximately 0.05 gram. These preparations also produce skin reactions and fever in human beings. The lethal substances resemble other toxins in that they are antigenic and are neutralized by the corresponding anti-toxin. The pneumococcus toxin is somewhat unusual in being type-specific. However, the large amount necessary to kill experimental animals, and the stability to heat sharply differentiate these substances from toxins such as those of diphtheria and tetanus. The relation between the symptoms produced by the toxin and those resulting from infection with the organism has not been clearly demonstrated. It is possible that insignificant amounts of these substances are produced by pneumococci and streptococci under artificial cultivation but that larger amounts are produced *in vivo* and do play a part in pneumococcus and streptococcus infections. The development of methods for producing, concentrating, and purifying these so-called toxins will lead to a better knowledge of their biological activity.

Like the streptococcus, the staphylococcus probably produces several toxins or poisonous substances which may usefully be investigated by chemical methods. The identity or separability of hemolyzing, necrotizing, and lethal toxins has not definitely been settled. Glenny and Stevens (1935) and Roy (1937) have described α and β staphylococcus toxins which differ in their ability to hemolyze human and rabbit red cells, to produce necrosis in the skin, and to kill susceptible animals. There are corresponding anti-toxins for these α and β toxins. The gastro-intestinal toxin described by Dack and his co-workers (1931) and Dolman (1934) apparently differs both in its chemical and biological properties from the hemolytic and necrotizing toxins of the staphylococcus. This substance is apparently non-antigenic and may not be a true toxin (Jordan and Burrows, 1933).

Some progress has been made in the purification and concentration of staphylococcus toxoid. Holt (1937) prepared toxin in a dialysate medium; and the formalin-toxoid from this toxin

was then precipitated with ammonium sulphate. The resulting product was free of the nitrogenous constituents of the medium but contained a carbohydrate derived from the agar used. Ramon, Boivin, and Richou (1936) have concentrated staphylococcus toxoid by precipitation with trichloracetic acid at pH 4.0.

b. Toxins of the Salmonella, Proteus, colon, and dysentery groups of organisms. Raistrick and Topley (1934) obtained from *Salmonella aertrycke*, by digestion of the acetone-extracted bacilli with trypsin and precipitation with 68 per cent alcohol, an antigenic fraction which produces somatic "O" agglutinins in the serum of immunized animals. As shown by Martin (1934) and Delafield (1934) this fraction is toxic for mice and rabbits. Herter and Rettger (1937) have also described toxic fractions obtained from *S. aertrycke*. Substances of a nature similar to that of Raistrick and Topley have been obtained by Boivin and Mesrobeanu (1937a) from members of the *Salmonella*, *Proteus*, and colon groups by extraction of the bacterial bodies with trichloracetic acid and precipitation of the extracted material with alcohol. These toxic substances are produced by the smooth but not by the rough variants and are independent of the presence of the H antigen. They contain from 3 to 5 per cent of nitrogen, small amounts of sulfur and phosphorus and give 20 to 40 per cent of reducing sugars on hydrolysis. An ether-soluble lipid component is split off by acid hydrolysis. Tests for peptide linkages and tyrosine are positive, but the substances are not precipitated by the ordinary reagents for proteins. This carbohydrate-lipid complex constitutes about 10 per cent of the bacterial bodies, kills mice in doses of 0.1 to 1.0 mg., produces only weak antitoxin, and is stable to heat in a neutral but not in acid or alkaline solutions.

The existence of a second but weaker endotoxin is indicated by the observation of Boivin and Mesrobeanu (1937a) that, after destroying the gluco-lipid complex by heating with dilute acetic acid, residual toxicity remains. This endotoxin, which is destroyed by tryptic digestion, is apparently a polypeptide, and is found in rough variants as well as in smooth.

Extending their observations, Boivin and Mesrobeanu (1937

b, c) demonstrated the existence of endotoxins in the bodies of Shiga and Flexner dysentery bacilli. These gluco-lipid complexes produce acute gastro-intestinal symptoms in experimental animals and are similar to the substances obtained from other gram-negative bacilli. Morgan (1936) has isolated and analyzed a specific nitrogenous polysaccharide from the Shiga bacillus. Other toxic fractions from dysentery organisms have been described by Olitski, Reibowitz, and Berman (1937).

Boivin and Mesrobeanu (1937c) have carried out a chemical separation of the endotoxin from the exotoxin in filtrates of cultures of Shiga dysentery bacilli. The exotoxin, which is formed by both R and S variants, is precipitated by trichloracetic acid at pH 3.5. After complete precipitation of the exotoxin, the endotoxin remains in solution and is recovered by precipitation with alcohol. In contrast to the endotoxin, the exotoxin is destroyed by tryptic digestion and by heat, has the general properties of a protein, and acts on the central nervous system but not on the gastro-intestinal tract.

This work on the Shiga dysentery toxin has been confirmed by Haas (1937a) who separated a heat-labile neurotropic exotoxin from a heat-stable endotoxin using the methods of Raistrick and Topley and of Boivin and Mesrobeanu. The endotoxin prepared by Haas is biuret-negative, gives a strongly positive Molisch test, and contains only 2.25 percent of nitrogen. It is slightly more active and may, therefore, be purer than the substances obtained by other investigators. Haas points out that since nutrient broth gives a protein precipitate with trichloracetic acid, the protein nature of the exotoxin has not been conclusively demonstrated.

2. Purification of bacterial toxins

The isolation of bacterial toxins in a pure state must depend upon the simplification of culture media and the perfection of chemical methods for separating the toxins from the constituents of the culture media and from proteins and other non-toxic products of the bacteria.

Although the plant toxin, ricin, was isolated as a protein by

Osborne, Mendel, and Harris in 1905 by fractionation with ammonium sulphate, the technical difficulties of separating bacterial toxins in an unaltered condition from the mixture of proteins, proteoses, and peptones with which they are associated in crude filtrates have not been so easily overcome. The simplest and generally most successful method has been precipitation of the toxin-containing protein fraction with acid, as applied to diphtheria toxin by Watson and Wallace (1924) and Locke and Main (1928), to botulinus and tetanus toxins by Snipe and Sommer (1928) and Sommer (1937), and to diphtheria, tetanus, and staphylococcus toxins and anatoxins by Boivin and Izard (1937). It is evident, however, from recent work that diphtheria toxin and possibly other toxins are damaged by precipitation with acid. Diphtheria toxin may be purified without alteration by adsorption on aluminum hydroxide and elution with phosphate buffer (Lindstrøm-Lang and Schmidt, 1930, and others), but some proteins, proteoses, and other constituents of the culture medium are adsorbed and eluted under the same conditions as the toxin. More extensive references to the literature on the purification of diphtheria toxin will be found in the paper by Eaton (1936a).

Using a new method which consists in precipitating the toxic fraction with the salts of aluminum and cadmium under carefully controlled conditions, Eaton (1936a) succeeded in obtaining highly purified, unaltered diphtheria toxin. It was produced in the proteose peptone medium of Wadsworth and Wheeler (1934). Pappenheimer (1937) obtained one of similar purity by simple ammonium sulphate fractionation of toxin produced in a medium made from hydrolyzed gelatin, amino acids, and accessory substances from liver extract.

Partial separation of toxin from bacterial proteins has been accomplished by fractionation with ammonium sulphate and fractional adsorption of the bacterial proteins on the colloidal hydroxides of magnesium or aluminum (Eaton, 1936a, Pappenheimer, 1937). One of the bacterial proteins is precipitated by 0.33 saturated ammonium sulphate solution at pH 7.0, and it is thus easily separated from the toxin which is precipitated be-

tween 0.4 and 0.6 saturation. A second bacterial protein precipitated at the same concentration of ammonium sulphate as the toxin may be separated by precipitating the toxin at pH 5.4 in a 0.33 saturated ammonium sulphate solution (Eaton, 1937b). Although the toxin is slightly damaged by this procedure it is the only successful method yet devised. The character of the

TABLE 1

Diphtheria toxin and anatoxin

INVESTIGATOR	M.L.D. OF BODY WEIGHT (GUINEA PIG)*	NITROGEN PER Lf UNIT*
	grams per kgm.	*mgm.*
Locke and Main (1928)	Not given	0.0006–0.0008
Eaton (1936a)	4.0×10^{-7}	0.00046–0.00055
Pappenheimer (1937)	4.0×10^{-7}	0.00046
Theorell and Norlin (1937)	Anatoxin	0.00088
Boivin and Izard (1937)	Anatoxin	0.00045

Other toxins

INVESTIGATOR	TOXIN	TEST ANIMAL	M.L.D. OF BODY WEIGHT*
			grams per kgm.
Osborne (1905)	Ricin†	Rabbit	5.0×10^{-7}
Sommer (1937)	Botulinus	Mouse	2.0×10^{-7}
Sommer (1937)	Tetanus	Mouse	1.0×10^{-6}
Eaton (unpublished)	Tetanus	Mouse	4.0×10^{-7}
Eaton (1936c)	Tetanus	Guinea pig	1.5×10^{-7}

* Calculated in some cases from figures given in other terms by the authors in order to make the results comparable.

† Not a bacterial toxin. Cited for comparison.

bacterial protein separated by differential adsorption on metallic hydroxides is not known at present.

Bacterial proteins in purified diphtheria toxin have been detected by precipitin tests with anti-sera against these proteins. Eaton used anti-serum prepared by injecting rabbits with washed whole diphtheria bacilli. Pappenheimer prepared anti-serum against the bacterial protein precipitated by one-third saturated ammonium sulphate; however, it seems likely that this anti-serum might fail to detect the bacterial protein which is pre-

cipitated at the same concentration of ammonium sulphate as the toxin (at 0.4 to 0.6 saturation). Estimation by the precipitin test of the amount of bacterial protein in purified toxin was made by titrating the antibacterial serum first against known amounts of bacterial protein free of toxin, and then against the preparation of purified toxin (Eaton, 1937b). The results indicate that in preparations containing the minimal amount of total protein per Lf unit, 10 to 15 per cent of the protein is bacterial precipitinogen and the rest toxin. The amount of bacterial protein may be reduced to 1 or 2 per cent by fractionation with acid as previously described, but not by simple fractionation with ammonium sulphate.

A summary of the more successful results of purification is presented in table 1. In all of these experiments the bacterial toxins have been separated from over 99 per cent of the nitrogenous impurities.

In two cases botulinus and tetanus toxins more active than the purest diphtheria toxin have been prepared. This is not surprising in view of the fact that these two toxins in the crude state are more active than diphtheria toxin. Diphtheria toxin and ricin are the only two toxins for which convincing evidence of purity has been advanced. Both are apparently proteins coagulable by heat.

3. Criteria of Purity

Since it has not been possible as yet to crystallize toxins, we must use criteria of purity which are not generally applied in organic or inorganic chemistry but which are of considerable value when applied to proteins with well-defined biological properties.

The attainment of a constant ratio of weight or nitrogen content to biological activity has been applied as a criterion of purity to diphtheria toxin and ricin. Karrer and his associates (1924) were unsuccessful in an attempt to separate from ricin, prepared by the method of Osborne, Mendel, and Harris, more active fractions by methods of adsorption or precipitation. Fractions showing less activity were sometimes obtained but these were probably

toxin altered by the chemical treatment. The nitrogen per Lf unit of diphtheria toxin has not been reduced below 0.00045 milligram by repeated precipitation with a variety of reagents, by fractional adsorption on metallic hydroxides or kaolin, or by repeated fractionation with ammonium sulphate (Eaton, 1936a, 1937b, Pappenheimer, 1937). It is possible that repeated chemical fractionation of a toxin may cause an amount of destruction that equals or slightly exceeds the further purification attained. In the separation of diphtheria toxin from bacterial protein by fractionation with acid, it can be demonstrated by the the precipitin test that impurities have been removed but the nitrogen per Lf is not reduced and may be, in certain instances, slightly increased because of partial destruction of the flocculating properties (Eaton, 1937b).

Measurements of physical and chemical properties such as optical rotation, molecular weight, precipitability by protein reagents, iso-electric point, and content of nitrogen and various amino acids have been used to characterize diphtheria toxin and ricin as proteins. The demonstration of the absence of impurities such as pigments, carbohydrate, proteose, peptone, and compounds containing phosphorus, from purified preparations of toxic protein constitutes evidence that the protein is the toxin. However, none of these tests proves that it is not mixed with another, atoxic, protein having otherwise similar properties.

Pappenheimer and Robinson (1937) have shown that the nitrogen per Lf unit of diphtheria toxin specifically precipitated by antitoxin corresponds to the nitrogen per Lf unit of the purest preparations obtained by chemical methods. Unless it proves possible to split off part of the flocculating protein without affecting the toxic properties, this is evidence that the purified preparations are almost pure toxin. Under the conditions of the experiment, bacterial protein detectable by anti-bacterial serum remained in the supernatant. The identity with the purified toxic protein of the substance precipitated by antitoxin from the crude preparation was not proved except by the close correspondence of nitrogen per Lf unit. Full proof would require that the experiment be performed with purified antitoxin.

Theorell and Norlin (1937) purified diphtheria anatoxin by cataphoresis in the Theorell apparatus and obtained a preparation containing about one and a half times as much nitrogen per Lf unit as diphtheria toxin purified by other methods. The authors conclude that their preparation is a pure protein because it travels as a unit in the electric field, but purer anatoxin has been prepared by purification of crude anatoxin and by detoxification of purified toxin with formaldehyde. Uniformity of motion in an electric field is not, therefore, a valid criterion of purity in this case.

Titrations of the equivalent weight of ricin done by Karrer and his associates (1924) indicated that their preparation consisted of higher and lower molecular fractions although it was not possible to separate these by any of the methods used. Even crystallization does not always guarantee the uniformity of a protein as shown by Hewitt (1936) for serum albumin. Thus far, apparently no studies of purified toxins or attempts to purify these substances by the ultracentrifugation method have yet been made. Pappenheimer (1937) gives the minimum molecular weight of diphtheria toxin as about 18,000.

4. Hydrolysis, digestion, and analysis of toxins

Up to the present time, chemical analyses of purified ricin and diphtheria toxin have yielded no clue as to the nature of the toxic structures. Karrer was unable to find any unusual grouping in ricin by analyzing the products resulting from tryptic digestion and hydrolysis with acid, although two kilograms of the purified protein were available for this study. Ricin contains cysteine. Eaton (1936a) found that diphtheria toxin gives negative nitroprusside and lead acetate tests for cysteine sulfur. Pappenheimer (1937) finds that his preparations of diphtheria toxin contain 0.75 per cent sulfur but give a negative nitroprusside reaction. It is probable, therefore, that the sulfur does not exist in the usual sulfhydryl configuration. Neither diphtheria toxin nor ricin contains phosphorus; they both contain about 16 per cent nitrogen. The high content of arginine and glutamic acid in ricin indicates that this protein has many free basic and acidic groups.

Diphtheria toxin apparently also contains much arginine as indicated by the strong Sakaguchi test. Tryptophane and tyrosine are present in both diphtheria toxin and ricin. Eaton (1936a) obtained weak qualitative reactions for tryptophane in 0.1 per cent solutions of diphtheria toxin, and Pappenheimer finds that a 1 per cent solution gives strong reactions for tryptophane. Diphtheria toxin contains 1.14 per cent of this amino acid as compared with 0.4 per cent in ricin.

With the possible exception of botulinus toxin, the endotoxins of the *Salmonella*, colon, and dysentery groups and certain of the toxic products of the hemolytic streptococcus, bacterial toxins are destroyed by digestion with proteolytic enzymes. This signifies that peptide linkages similar to those found in proteins are essential to the activity of the toxin. It does not necessarily mean that the chemical group or groups in toxins which poison living cells are polypeptide chains. The numerous examples of enzymic destruction of toxins will not be reviewed in detail; some are referred to in other sections of this paper, but the resistance of certain toxins to digestion will be discussed more specifically here.

The resistance of botulinus toxin to digestion has been demonstrated by Bronfenbrenner and Schlessinger (1924), Schubel (1923), Tani (1934), and others. Nelson (1927) found the toxin to be intimately associated with a globulin from the bacterial cell. Digestion with pepsin removed this globulin without destroying the toxin. Botulinus toxin may not be, however, absolutely refractory to digestion. The fact that the minimal lethal dose by mouth is about a hundred times as great as by injection indicates either that much of the toxin is destroyed in the gastrointestinal tract or only a small part is absorbed. In at least one instance partial destruction by pepsin has been observed (Tani, 1934). Karrer (1924) found that ricin is digested very slowly by trypsin. Of a 50 gm. lot of the toxin only two-thirds, as determined by parallel measurements of amino nitrogen and toxicity, was digested by 40 cc. of pancreatic juice in five months.

Resistance to tryptic digestion in the case of the endotoxins of the *Salmonella*, colon, and dysentery groups of organisms is

attributable to the fact that these substances are complex compounds of carbohydrate and lipid. This may also be true of one of the scarlatinal toxins.

PART III. THE CHANGES PRODUCED BY THE ACTION OF VARIOUS PHYSICAL AND CHEMICAL AGENTS ON TOXINS

The demonstration that a given chemical or physical treatment detoxifies a toxin is of little significance unless the nature of the changes produced in the toxin can be determined or deduced from the experiment. We shall consider here only those studies in which effects on other properties besides toxicity have been examined.

1. Denaturation

Most toxins are destroyed by the action of heat, strong acid, or alkali. From observations on crude unconcentrated toxins, the nature of the changes induced is not clear. It has been assumed that the lability of some toxins to heat is analogous to the heat-lability of many proteins. The demonstration that purified and concentrated diphtheria toxin is coagulated by heat and denatured by acid and alkali lends support to the view that the destruction of toxins by these agents is actually a denaturation of protein.

Although little is known about the chemical changes which occur when proteins are denatured, it is possible to correlate alterations in biological properties with denaturation. Partial denaturation, without loss of solubility at pH 7.0, apparently affects first those properties of diphtheria toxin designated by Ehrlich as the "haptophore groups." If the denaturation is carried farther, the protein is coagulated and all of the biological properties of the toxin are destroyed. According to the degree of denaturation, diphtheria toxin loses in various degrees its ability to combine and flocculate with antitoxin, its antigenicity, and its toxicity, as shown by Eaton (1936b). An increase in the time required to flocculate with antitoxin is probably the most sensi-

tive indicator of denaturation, and increased Kf[3] is apparent before changes in solubility or other gross evidence of denaturation. The length of time toxin is exposed to denaturing agents affects in a corresponding degree its Kf and antigenicity. Denatured toxin or toxoid of low antigenicity may be separated from unaltered toxin or toxoid of higher antigenicity by chemical treatment of partially denatured preparations. Papers bearing on this subject have been reviewed previously (Eaton, 1936b, 1937b).

The loss of toxicity which accompanies denaturation may be considered either as a decomposition of the toxic groups or as an impairment of the ability to combine with or attack susceptible cells. Possibly heat and high concentrations of hydrogen or hydroxyl ions destroy the toxic groups and at the same time denature the protein and destroy its antigenic properties. It appears, however, that at lower temperatures in neutral or slightly alkaline solutions the rate of toxoid formation or modification of the toxicity is more rapid than the rate of denaturation, while at higher temperatures, and especially in pH ranges above 8.0 and below 5.0, denaturation is more rapid than formation of toxoid. The range of pH for maximum stability varies, of course, for different toxins. Diphtheria toxin is most stable in neutral solution, while botulinus toxin is apparently most stable in acid solution and relatively unstable in an alkaline solution.

The so-called reversible detoxification of diphtheria toxin by acid, as reported by earlier investigators, is probably the result of a combined precipitation and coagulation. Later investigations have shown that the acid-detoxification is only partially reversed by bringing the pH of the solution back to neutrality. It is also possible that the acidified toxin solution precipitates proteins at the site of injection, thus hindering the spread of the toxin.

2. Agents which produce destruction, denaturation, or modification

Many reports dealing with the action of chemicals on bacterial toxins have failed to state what effects were produced on the

[3] Kf is the time required for flocculation of a mixture of toxin and antitoxin in optimal or equivalent proportions. Since this depends on the concentration of toxin and antitoxin in the equivalence mixture, the Kf of different preparations of toxin must be compared by using solutions with the same number of Lf units per cc.

antigenicity or combining power for antitoxin. A review of the earlier work has been published by Bacher (1927). More recently Schmidt (1932) has carried out an extensive investigation of the effects of many aliphatic, aromatic, and alicyclic compounds on the toxicity and Lf value of diphtheria toxin. Only formaldehyde, acetaldehyde, glyoxal, glucose, furfural, hexamethylene tetramine, and certain halogenated hydrocarbons reduced the toxicity without a corresponding diminution in the Lf values. All the other compounds studied either were inert or diminished the Lf in parallel with the toxicity. Judging from the results of Schmidt and other investigators, it seems that the effect of most chemical compounds on bacterial toxins is very similar to that of heat. Many of the substances which destroy toxins also denature proteins.

Von Groer, Altenberg, and Lille (1935) have reported that diphtheria toxin is destroyed by the ortho- and para-dihydroxyphenols but not by the meta-compound. The authors imply that there may be a stereochemical relationship involved, but the o- and p-compounds are much more reactive with a variety of substances than is the m-compound. Schmidt (1932) found that most of the aromatic phenols rapidly destroy diphtheria toxin.

Many of the toxins from anaerobes are quite readily destroyed by oxidation. Diphtheria toxin is not particularly sensitive to mild oxidation or reduction (Hewitt, 1930). Scarlatinal toxin may be in part modified to toxoid by oxidation. Cowles (1936) has shown that cysteine catalyzes an oxidative modification of tetanus toxin. Atmospheric oxygen is used up in the process. The detoxified preparations are antigenic and retain 20 to 50 per cent of their combining power for antitoxin. The work of Halter (1936) indicates that tetanus toxin, when it is diluted in sodium chloride solution, may be destroyed by oxidation. The recent observation of Jungeblut (1937) that about 2 M.L.D. of tetanus toxin may be inactivated by ascorbic acid in amounts between 0.5 and 10 mg. is possibly also connected with an oxidative change. Lippert (1935) finds that methylene blue in the presence of light brings about the destruction of tetanus toxin. He believes that the reaction is an oxidation.

Studies of the photodynamic action of methylene blue on other toxins have been reported by Lin (1936) and Li (1936). Lin states that diphtheria toxin modified by the photodynamic action of methylene blue is apparently more antigenic than formol-toxoid. However, the detoxification was not complete, and, since eight injections were used for immunization, a considerable immunity could have been produced by the residual toxin. No reports of the effect on flocculation were given. Li was able to remove completely the hemolytic, dermo-necrotizing, and lethal properties of staphylococcus toxin by the combined action of methylene blue and light. The toxoid thus produced was equal in antigenicity to formol-toxoid and alum precipitated formol-toxoid.

3. The action of formaldehyde

Formaldehyde apparently acts directly on the toxic groups without affecting other parts of the toxin molecule which are concerned with antigenicity and combining activity. The nature of the chemical reaction or series of reactions that occur may be successfully studied only with highly purified toxin because many other substances in crude toxin also react with formaldehyde.

Diphtheria toxin seems best suited for studies on the process of toxoid formation, because it is easy to measure the amount of antigenic material by the flocculation test and because the toxin may be obtained in a relatively pure state. Bunney (1931) reported that diphtheria toxin purified by acid precipitation could not be detoxified by formaldehyde without destroying the antigenic properties. Others have not confirmed Bunney's results. Eaton (1937c) observed that an excess of formaldehyde in an alkaline solution impairs the flocculating, combining, and immunizing properties of purified toxin during modification to toxoid. The presence of small amounts of nitrogenous impurities in partially purified toxin will markedly affect the action of formaldehyde and prevent destruction.

For detoxification in a solution at pH 6.0 there is required a concentration of formaldehyde one hundred times as great as that sufficient to modify purified toxin to toxoid in a solution at

pH 8.6. Follensby and Hooker (1936), using diphtheria toxin partially purified by acid precipitation, showed that the reaction between formaldehyde and toxin to form toxoid has the characteristics of a unimolecular reaction. The velocity constant of the reaction is stated to be directly proportional to the concentrations of hydroxyl ions and formaldehyde.

Hewitt (1930) has pointed out that the reaction between formaldehyde and toxin to form toxoid is slow and irreversible while that between formaldehyde and the free amino groups of proteins, polypeptides, or amino acids is rapid and reversible. With both crude and purified toxin the amount of formaldehyde required for detoxification in a reasonable time is not greater than the theoretical quantity necessary to combine all of the free amino nitrogen (Eaton, 1937c). In the reaction which occurs in the Sørensen titration a large excess of formaldehyde (about 60 times the theoretical amount) must be added to combine with all of the amino groups. From these facts it is obvious that the reaction which occurs in toxoid formation is not the ordinary reaction between formaldehyde and amino groups.

Compounds of formaldehyde or acetaldehyde and ammonia form toxoid more slowly than do the free aldehydes. Aldehyde bisulphites modify diphtheria toxin to toxoid slowly and incompletely (Eaton, 1937a). Wadsworth, Quigley, and Sickles (1937) have observed that the addition of histidine in a quantity sufficient to combine all of the formaldehyde in a mixture with toxin prevents the formation of toxoid. Apparently the affinity of the toxic group for formaldehyde is greater than that of ammonia or amino groups but less than that of the bisulphite ion or the imidazole group of histidine.

Purified diphtheria toxoid contains about two-thirds of the free amino nitrogen found in purified toxin, and the bound amino nitrogen in toxoid is not liberated by removal of the free formaldehyde (Eaton, 1937c). Wadsworth, Quigley, and Sickles (1937) were able to detect only minute amounts of amino nitrogen, or none at all, in diphtheria toxin partially purified by ultra-filtration. Pappenheimer (1937) found about 1.2 per cent, and Eaton found over 2.0 per cent of free amino nitrogen in their purified

preparations. In the experiments of Wadsworth, Quigley, and Sickles about one-tenth of the formaldehyde from a 0.22 per cent solution disappeared during the complete conversion of toxin to toxoid over a period of 20 days. This quantity of formaldehyde appears to be greater than the equivalent of all the amino groups in both toxin and impurities.

The combination of formaldehyde and amino groups is, of course, not necessarily the reaction concerned in detoxification. Present data are not accurate enough to indicate whether all of the formaldehyde used up during detoxification combines with amino groups, but measurements of this sort are not impossible with highly purified and concentrated toxin. Follensby and Hooker (1936) suggest that the formation of toxoid from toxin may be a reaction catalyzed by formaldehyde and hydroxyl ions. If this is true, the disappearance of formaldehyde to combine with amino nitrogen and other groups is only an incidental reaction. Recently Goldie (1937) has studied the action on crude and partially purified diphtheria toxin of ketene, an acetylating agent that combines directly with free amino groups. In the various samples of crude and of purified toxin 30 to 50 per cent of the amino groups were combined after 10 to 25 minutes' action of the ketene, and the toxicity had been reduced to 1/6 to 1/300 of the M.L.D. in the original sample. At this stage of the acetylation the toxin flocculated with antitoxin, indicating that a partial modification of toxin to toxoid had occurred. With more prolonged action of the ketene, further combination occurred and both flocculating ability and toxicity were destroyed.

Formaldehyde-toxoid is more stable to denaturation than toxin (Eaton, 1937c). It is not at present known whether the acquirement of increased stability is directly connected with loss of the toxic properties. Other proteins also become less subject to denaturation, as judged by decrease in solubility, after the action of formaldehyde. The changes which occur are not understood as yet. The fact that purified toxin and toxoid have the same optical rotation suggests that the optically active atoms adjacent to the peptide linkages may not be affected in the change to toxoid.

With certain proteins, such as casein, formalin produces polymerization, and some investigators have taken the view that toxoid is a polymerization product of toxin. However, in the case of diphtheria toxin, one Lf unit of toxin is modified by the action of formaldehyde to one Lf unit of toxoid, and there is no appreciable change in the nitrogen per flocculating unit. Consequently, polymerization of two or more molecules of diphtheria toxin to form toxoid could only be possible if all the groups which combine with antitoxin remained free. In the case of staphylococcus toxin, the formation of toxoid by the action of formaldehyde is accompanied by a reduction in the combining power for antitoxin to approximately one-half. An indication of a difference in the combining powers of diphtheria toxin and toxoid is found in the work of Madsen, Jensen, and Ipsen (1937) who studied the combination *in vivo* of injected toxin or toxoid with antitoxin in the blood of actively and passively immunized animals. Their results indicate that diphtheria toxin binds twice as much antitoxin *in vivo* as *in vitro*, while toxoid binds the same amount of antitoxin *in vitro* as *in vivo*.

Although denatured toxin of less combining power and toxicity may be separated by chemical means from a mixture with unaltered fully active toxin, the separability of toxin from toxoid has never been demonstrated. The progressive formation of toxoid from toxin may be a process affecting step-wise all the molecules of toxin at once so that there is not at any time an equilibrium between "completely toxic" toxin and "completely atoxic" toxoid. The fact that the complete reaction is irreversible points against the existence of such an equilibrium. There may however, be a reversible equilibrium between molecules modified to different degrees before complete detoxification. This is indicated by some observations of Wadsworth, Quigley, and Sickles (1937) who found that, following the removal of formaldehyde by ultrafiltration from partially modified toxin, the toxicity increased when the preparation was incubated but not when it was kept in the cold room. This implies a partial reversal of the reaction at an intermediate stage.

4. The action of soaps, lipids, and sterols on bacterial toxins

Vincent (1926) observed that 0.2 to 1.0 per cent solutions of bile and soaps neutralize several hundred M.L.D. of tetanus and other toxins. This author termed the effect a masking of toxicity rather than an inactivation because the reaction was partly reversible. Precipitation of the palmitic acid of the soap with hydrochloric acid liberated enough tetanus toxin to kill guinea pigs, but the killing doses were considerably larger than the M.L.D. of the original toxin.

Larson and Nelson (1924) attributed the detoxifying effects of sodium ricinoleate on diphtheria and tetanus toxins to the property of this substance of forming colloidal aggregates capable of adsorbing other colloids. Larson and Halvorson (1925) observed that the toxin-soap mixture was dissociable. Dilution caused it to become toxic and the firmness of combination increased with time. Bayliss (1936) found that sodium ricinoleate and sodium chaulmoograte are the most effective detoxifying soaps, a 1 per cent solution neutralizing about 35 M.L.D. of diphtheria toxin. Other soaps of chemical composition similar to the ricinoleates and chaulmoograte were less effective. Unsaturated soaps were generally more active than salts of the saturated fatty acids; bile salts were least effective.

A somewhat different effect of the salts of fatty acids on diphtheria toxin has been observed by Schmidt (1932). The lower members of the series are inactive but, beginning with the fatty acid containing eight carbon atoms in the chain, a destruction of toxicity, flocculating, and immunizing properties is produced by solutions as dilute as hundredth normal acting over a period of several weeks. In these experiments the effect of pH was controlled. Schmidt also observed that a large excess of ricinoleate was necessary to detoxify diphtheria toxin in 24 hours. Contrary to the results of Larson and his collaborators who claimed that their preparations were good antigens, Schmidt states that ricinoleate-toxin has only weak antigenic properties which may be due to traces of free toxin.

Fixation of toxin at the site of injection as a result of previous

adsorption on colloidal particles has been advanced by Ramon and his collaborators (1937b) as the explanation for the neutralizing effects of lanolin on diphtheria and tetanus toxins. By emulsification with 3 or 4 grams of lanolin, 40 M.L.D. of diphtheria toxin or 200 M.L.D. of tetanus toxin were rendered innocuous. Addition of cholesterol diminished the neutralizing effect of lanolin on tetanus toxin. Removal of the lanolin by extraction with acetone, toluol, or chloroform liberated part of the toxin. Mixtures harmless to guinea pigs were found to be toxic for rabbits. Local fixation of the toxin injected with lanolin was demonstrated by studies on the rate of distribution of the toxin in the body of the animal. One or two injections of the toxin-lanolin emulsion are said to produce better immunity than the injection of an equivalent amount of anatoxin. Ramon's results have been confirmed by Eisler and Gottdenker (1937). Using a solution of cholesterol in olive oil, these investigators found that the degree of detoxification of diphtheria toxin depends on the relative volumes of toxin and oil and the length of time these are shaken together. The results apparently depend on surface effects in the droplets of oil. Aqueous emulsions of cholesterol do not affect diphtheria toxin. According to Ramon cholesterol is less effective than lanolin as a detoxifying agent. Schwartz (1936) has reported that the hemolytic, necrotizing, and lethal properties of staphylococcus toxin are markedly diminished by shaking with olive oil.

The colloidal phenomena just described differ in several ways from the strictly chemical effect of formaldehyde on toxin. Relatively enormous amounts of the soap or lipid (in the proportion of about 100,000 parts to 1 of toxin) are required to detoxify. The effects on the antigenic and combining properties of the toxin are not at present clear. Soaps apparently affect the properties of the toxin in a way which is similar in part to denaturation and in part to combination with antitoxin. Possibly partial denaturation of toxin is produced by surface effects in emulsions just as shaking with air denatures purified and concentrated diphtheria toxin (Pappenheimer, 1937), and proteins generally. On the other hand, some of the effects are at least

partially reversible. The high antigenicity of lanolin-toxin mixtures seem to preclude denaturation. Probably some of the agents act by delaying absorption of the toxin at the site of injection. Others, such as soaps, may act by binding those parts of the molecule which attach the toxin to susceptible cells, antibody-producing cells, or antitoxin. True modification of toxin to toxoid probably does not occur in any case.

5. The effects of various chemicals on toxins and other substances that act on red blood cells

Substances that hemolyze or agglutinate red cells are of interest in connection with the chemistry of toxins, because factors affecting the combination of active substance and susceptible cells may be studied. Staphylococcus toxin and plant toxins such as ricin also produce toxic effects *in vivo*. Others acting only *in vitro* are pneumococcus hemolysin, tetanolysin, and the crystalline protein concanavalin A (Sumner and Howell, 1936), which agglutinates red cells and precipitates a variety of carbohydrates and lipids.

Hypertonic salt solutions inhibit hemolysis by immune serum and complement and also by bacterial hemotoxins. Rigdon (1937) finds that the combination of staphylococcus hemotoxin with rabbit red cells is prevented by 6 per cent sodium chloride solution. Avery, Rigdon, and Johlin (1937) report that magnesium sulphate and several salts of sodium and potassium inhibit hemolysis by staphylococcus toxin. The production of skin necrosis by this toxin is also inhibited by hypertonic solutions of sodium chloride, magnesium sulphate, and lithium chloride. The latter salt, however, does not prevent hemolysis. Smith (1937) has found that glycerol, ethylene glycol, sucrose, and glucose, diminish the necrotizing and lethal properties of staphylococcus toxin but have no effect on the titration of toxin by hemolysis of red cells. Weinstein (1937) reports that the hemolysis produced by colonies of streptococci and staphylococci growing on blood agar is inhibited by adding lecithin to the medium. Cholesterol prevents this inhibition of hemolysis. This recalls antagonistic action of cholesterol on the neutralization of tetanus

toxin by lecithin as observed by Ramon. Salts, lecithin, and polyhydroxy-alcohols may act directly on the hemotoxin, or they may alter the surface of the cells so as to prevent attachment of the toxin.

The identification of at least one chemical group associated with the activity of pneumococcus hemotoxin has been accomplished by Cohen and Shwachman (1936), and Shwachman, Hellerman, and Cohen (1934). Pneumococcus hemolysin is inactivated by oxidation and reactivated by reagents that can restore free thiol groups; therefore the lytic activity is associated with the presence of sulfhydryl groups in the preparation. Reversible oxidation is produced by a variety of agents, and zones of reduction potential in which the hemolysin is active or inactive have been defined. The iodoacetate ion which inactivates certain enzymes also inactivates the hemolysin, but does so reversibly.

Cohen, Shwachman, and Perkins (1937) have examined the effects of various sterols on pneumococcus hemolysin. Irreversible inactivation is produced by cholesterol and coprostenol which contain double bonds and are precipitable by digitonin. The saturated sterol, coprostanol, is less active and various sterols not precipitated by digitonin are practically without effect. Binding of the hydroxyl group by esterification as in cholesteryl acetate also removes the inhibitory action. These observations are similar to previous ones on tetanolysin and saponin. Diphtheria toxin differs from these substances in being neutralized both by sterols and their esters. This indicates that a different mechanism is involved (Eisler and Gottdenker, 1937).

Active pneumococcus hemolysin combines rapidly with red cells and with cholesterol. Inactive hemolysin does not combine with red cells nor, apparently, with cholesterol, because after treatment with the latter the hemolysin may be reactivated as usual. It remains to be seen whether or not this indicates that the active hemolysin combines with cholesterol in the red cells. Should it do so, then the attachment could not occur through the sulfhydryl groups because these apparently remain free

(positive nitroprusside reaction) in the hemolysin after inactivation with cholesterol. At any rate, the state of oxidation of the sulfhydryl groups apparently conditions the affinity of other, unknown groups in the lysin for sterols and for red cells. The actual lysis of the red cells may be due to enzymic properties of the hemolysin. The loss of hemolytic power is apparently not accompanied by a loss of the ability to combine with antibody. Hull (1936) found no change in the combining capacity for antibody of hemolytic extracts of the pneumococcus after storage in the ice-box for a length of time sufficient to cause a marked decrease in the hemolytic property.

6. The effect of vitamin C (ascorbic acid) on diphtheria toxin in vitro and in vivo

The effect of vitamin C on poisoning with diphtheria toxin has been studied extensively since Greenwald and Harde (1935), and Jungeblut and Zwemer (1935) observed that feeding vitamin C to guinea pigs increased their resistance to 1 or 2 lethal doses of toxin. The effect of vitamin C on toxin *in vitro* is of doubtful significance. The investigators just cited reported that up to 10 M.L.D. of toxin were inactivated by 10 mg. of ascorbic acid per M.L.D. (The M.L.D. of pure diphtheria toxin is 0.0001 mg. or less.) Grooten and Bezssonoff (1936) state that 100 mg. partly detoxify 4 M.L.D. Hanzlik and Terada (1936) observed that neutralization of toxin was absent or irregular in alkaline solutions of vitamin C; and they found no protection of pigeons by vitamin C against diphtheria toxin. Torrance (1937b) reports that heated and unheated crude toxin catalyzes the oxidation of vitamin C in lemon juice, an effect that may be due to porphyrins in the crude filtrate. Lemon juice has no effect on the toxicity or Lf value of the toxin. Sigal and King (1937b) find that properly neutralized and buffered solutions of vitamin C do not inactivate diphtheria toxin when oxidation is inhibited by diethylthiocarbamate. The effects of vitamin C on diphtheria toxin *in vitro* may be attributed to oxidation and acidity.

The injection of sublethal doses of diphtheria toxin in guinea pigs causes a depletion of vitamin C from the suprarenals, pan-

creas, and kidneys (Lyman and King, 1936; Torrance, 1937a; Haas, 1937b). Lyman and King observed an increase or a decrease of vitamin C in the liver, depending upon the dose of toxin and the amount of vitamin given daily. Torrance reports that injection of small amounts of toxin causes a mobilization of vitamin C in the suprarenal gland, but Haas was unable to confirm this. Sigal and King (1937a) have studied the mode of action of vitamin C in diphtheria intoxication. Injection of sublethal doses of toxin into animals on a diet deficient in vitamin C produced a degeneration of the islets of Langerhans, a hyperglycemia, and a low glucose tolerance. The effect was less marked in animals on an adequate diet, but the amount of vitamin necessary for a maximum effect on the intoxication was much larger than that necessary to protect against scurvy and maintain the normal growth rate.

CONCLUSION

The last five years have witnessed definite advances in our knowledge of the mode of production and the nature of bacterial toxins. The development of media containing relatively simple nitrogenous substances of known composition, such as those used for the cultivation of *Corynebacterium diphtheriae* and *Staphylococcus aureus*, will undoubtedly make possible more exact chemical studies of toxins. The complex conditions under which toxins are formed can be worked out most easily when the constituents of the media are known. Among the important factors affecting the formation of toxins are conditions related to oxidation-reduction systems. Many other factors have not yet been clearly defined in their relationship to the mechanism of toxin formation.

The ease with which various toxins are liberated from the bacterial cells differs greatly. Some toxins such as that of *C. diphtheriae* appear to be secreted in a readily soluble form by the bacteria. Others are liberated only by death and autolysis of the cells. The view that toxins are formed by enzymic degradation of proteins or peptones in the culture media is becoming less tenable as more toxins are produced in media containing

substances not much more complex than the amino acids. It is possible, however, that some of the bacterial poisons, such as the enterotoxin of the staphylococcus and the hemolysin of the streptococcus, may be metabolites or products of the action of bacterial enzymes.

At the present time, the only bacterial toxin which has been isolated in a state approaching purity is diphtheria toxin. The concentration and partial purification of others by various chemical procedures has assisted in the study of bacterial products with poorly defined biological properties. Chemical studies of the toxic products of the hemolytic streptococcus have led to the discovery that this organism forms a host of substances, each having some of the characteristics of a toxin. In the separation of the endotoxin from the exotoxin of the Shiga dysentery bacillus it was shown that the gastro-enteric endotoxin is a carbohydrate-lipid complex, while the neurotropic exotoxin is a protein. This is an advance which may eventually enable us to differentiate endotoxins from exotoxins on a chemical basis.

Attempts to demonstrate the existence of a toxic group in toxins by chemical analysis have so far been unsuccessful because of the difficulty of obtaining toxins in a pure state and in sufficient quantity. Both ricin (a plant toxin) and diphtheria toxin appear to be proteins which cannot be further degraded without destroying their biological activity. There is at present no evidence that these are conjugated proteins containing a prosthetic group which is responsible for their physiologic activity. However, it is possible that other toxins, such as those of *Streptococcus hemolyticus, Clostridium botulinum,* and the endotoxins of the *Salmonella,* colon, and dysentery groups, may prove to be non-protein substances with some unique chemical configuration which gives them their toxic properties.

Studies on the action of various physical and chemical agents on toxins have begun to yield suggestive data. Toxins are apparently affected in three different ways: (1) by agents which produce denaturation or coagulation, (2) by agents (soaps and lipids), which reversibly mask the toxic and biologic properties, and (3) by agents (certain aldehydes, halogen compounds, and oxidizing

agents) which produce modification to toxoid. Further investigations with purified toxins and chemical reagents classified into these three groups will doubtless yield important information on the nature of toxins.

Many toxins produce pharmacologic effects resembling those of the alkaloids. Toxins differ, however, from ordinary poisons in their tremendous activity and in the properties connected with antigenicity and the ability to combine with antitoxins and with susceptible cells. The association of antigenicity and great toxicity may be more than incidental. It is not unlikely that the same property or chemical grouping which causes an immunologic response when the toxin is injected may also bring about a selective combination with susceptible cells, just as an enzyme combines selectively with certain substrates. The pharmacologic effect would then follow by the action of other parts of the toxin molecule. From this it would appear that the nature of toxins as proteins or protein-like substances may hold the secret of their most characteristic biological properties.

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[4]In preparing this review, the author has not attempted to cover every paper having a bearing on the subject. Enough references are given to illustrate each point but there are many other papers on similar work which have not been cited. Generally the most recent papers have been reviewed without regard to historical development or priority. The papers cited usually contain adequate references to earlier work.

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SEROLOGICAL RELATIONS AMONG SPORE-FORMING ANAEROBIC BACTERIA

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Immunochemistry in recent years has offered new proofs and better criteria for the specificity of antigen-antibody reactions (134, 174, 175, 207). These advances have strengthened the position of serologists by providing a firmer basis for their examination of biological materials, such as bacterial constituents. Remarkable examples of group- and species-specificity have been found, and in certain instances the information has been useful in bacterial classification.

It is the purpose of this review to present and evaluate the serological data relating to the spore-forming anaerobes[1] and to discuss the relationships disclosed and their bearing upon the taxonomy of these organisms. One must admit, however, that the serological relationships are not always in harmony with the present taxonomic schemes. Examples will be given in which the serological evidence may require consolidation of species that are at present listed separately. In other instances, the evidence either confirms the present species or reveals subdivision of them. For the present, then, it can only be claimed that the serological approach has discovered a certain measure of order, and that it does aid in the differentiation of the closely related spore-forming anaerobes.

The subject-matter of this review will be discussed under the

[1] Comparatively little is known concerning the serology of the non-spore-forming species, although several papers on this topic have appeared lately: 9–13, 59–61, 79, 80, 145, 303, and 364.

headings: *a* Toxigenic anaerobes and their toxin specificities (including the problem of atoxic strains); *b* Agglutination reactions; and *c* Precipitin and complement fixation reactions.

THE TOXIGENIC ANAEROBES AND THEIR TOXIN SPECIFICITIES

The toxins produced by certain of the spore-forming anaerobes have long been recognized as specific substances, though of unknown composition. Physiological evidence of this specificity is found in the consistent action in the animal body of such toxins as tetanospasmin and the neurotoxin of *Clostridium botulinum*.[2] The specificity of the toxin-antitoxin reaction was employed in the recognition of an anaerobe by Roux as early as 1888 (271). Through the World War period, when discovery of new anaerobes was abnormally stimulated, it became almost a routine to test for toxin neutralization with antitoxins of the most probable group relatives before a newly isolated strain was identified or described as new (52, 55, 168, 200, 277, 359, 360). In polymicrobial infections, like gas gangrene, proof of the etiologic agent was often obtained by "animal protection experiments" (70, 144, 176, 177, 263, 330). And finally, the controversy as to whether "Rauschbrand" of animals was comparable to gas edema in man, and caused by the same organism, was settled largely by study of the toxin specificities of *Clostridium chauvoei* and *Clostridium septicum* (55, 107, 169, 178).

Partly because the toxins are usually produced in complex nitrogenous media, the problems of the purification and nature of these substances are difficult to solve. Nevertheless, considerable efforts are being made to develop methods for purifying,

[2] Throughout this paper problems of nomenclature have been ignored so far as possible. In general, the name first proposed has been employed in direct reference to the discovery of an organism; in section headings and in later discussion that name, as adapted to placement in the genus *Clostridium*, has been used for all commonly accepted members of the genus. For the less familiar *Clostridium* species and particularly those whose familiar names have thus been displaced, synonyms have been given in parentheses following the first citation of the species. Whenever inclusion of an organism in *Clostridium* would involve the creation of a combination of names new to the literature, the transfer has not been made; in such cases, the original name in *Bacillus* has been retained for clarity of reference. This policy has seemed to the authors preferable to debating and deciding specific problems of nomenclature on this occasion.

preserving, and characterizing the principal toxins and antitoxins (19–24, 33, 78, 101–104, 129, 148, 152–154, 158, 204, 205, 290, 293, 298–300, 312, 313, 323, 336, 337, 339, 371). Recently two agencies, the Inter-governmental Conference on Biological Standardization and the Permanent Standards Commission of the League of Nations Health Organization, have undertaken to define international units for toxin and antitoxin preparations for tetanus and the gas gangrene organisms (1, 93, 94, 152, 153, 193, 204, 205, 337). The work of the League Committee has been directed primarily toward the definition of units and the investigation of the accuracy of titrations of toxin-antitoxin preparations. The State Serum Institute at Copenhagen is the depository for the standards. As the United States is not a member of the League, it has no official part in determining the international policy; but it has coöperated in conferences and in making tests. Definitions of the United States equivalents of the International units are made by the National Institute of Health. In some cases, the same units have been adopted, for example, that for oedematiens antitoxin (23); in other cases, a pre-existing American unit has been defined in terms of the International unit, for example, that for tetanus antitoxin (193).

Enough is known about the differentiation of the toxins of the principal anaerobic species to permit a discussion of these specific substances as a basis for the typing of the toxigenic organisms.

The toxins of Clostridium tetani, Clostridium septicum, and Clostridium histolyticum

Clostridium tetani presents a comparatively simple case of toxigenicity. Its toxin was discovered by Kitasato in 1889 (166), and the specific antitoxin by von Behring and Kitasato in the following year (15). The pharmacological action of the toxin indicates the presence of at least two factors: tetanospasmin and tetanolysin (87, 118, 181, 203, 258, 326, 335). For purposes of production of antitoxin, however, no distinction of these factors is made, the cell-free filtrate of a culture being used either directly, or modified by iodine (329), formol (278), or in combination with alum, tapioca, lanolin, etc. (255–257). Furthermore, experience has shown that a protective serum can be made

from any strain of true *C. tetani*. For this reason the toxin is called monotypic, a point of particular interest in view of the subdivision of the species into serological types by other reactions. The question of the mode of absorption and action of tetanus toxin has recently been reopened; but there is not as yet agreement upon the route of transfer of the toxin to the central nervous system (2–6, 74, 90).

Clostridium septicum (*Vibrion septique; Bacillus oedematis maligni*) also produces a toxin which has no type-specificity, although sub-types on the basis of agglutination do exist within the species. The general serological properties of the toxin are known (19, 20, 71, 158, 264, 266, 338, 348). The pathology of the extremely rapid death of animals injected intravenously with the toxin has been explained recently (245). A specific action of the toxin on the heart muscle, causing Zenker's degeneration, is the immediate cause of death, although lesions also occur in the kidney, spleen, and other organs.

Clostridium histolyticum also produces a toxin which is monotypic for the species but is apparently a complex of myolytic and hemolytic factors. The organism is unique in its myolytic action, a property which has been made use of in the Connell method (54) for the lysis of cancerous tissue. The efficacy of this treatment has been denied (117, 250, 272); but its proposal has at least led to a more thorough study of the proteolytic enzyme system, upon which the myolytic action probably depends (309, 340). It is peculiar that the red blood corpuscles remain intact in the residue of lysed tissue (123, 362). In culture, hemolysin is produced under proper conditions, but not in proportion to the general potency of the toxin as determined by the minimal lethal dose (228, 309). The preparation of the toxin and antitoxin and their standardization have recently been investigated (24, 153, 337).

The toxin of Clostridium oedematoides (*Clostridium oedematis sporogenes; Bacillus sordellii*)

A new species of the gas-gangrene group was discovered by Sordelli in 1922 (301). Because of its resemblance to *Clos-*

tridium oedematiens in pathogenicity in a human case and to *Clostridium sporogenes* in culture, it was called *Clostridium oedematis sporogenes*. The name, being a trinomial, was objectionable and was changed to *Bacillus sordellii* by Hall and Scott (127). Meanwhile, Meleney, Humphreys and Carp (219) discovered a *Clostridium oedematoides*, resembling *C. oedematiens* and *C. septicum* but distinguishable from both by toxin neutralization tests. The following year the toxins of *C. oedematoides* and *B. sordellii* were found to be identical; *C. oedematoides* was declared invalid and *B. sordellii* considered the preferred name for the species (125, 150). Agglutination tests have also indicated that the two organisms are identical (126). However, since *Clostridium* is now the widely accepted genus for the spore-forming anaerobes, *C. oedematoides* would be valid, and it has been retained by Hauduroy *et al.* (131) in the new French "*Dictionnaire des bactéries pathogènes pour l'homme, les animaux et les plantes.*" Nevertheless, the combination: *B. sordellii* or *Clostridium sordellii*, according to the choice of the user, continues to be in favor in this country.

Further study of the toxin-antitoxin reaction of this species has disclosed an unforeseen relationship to another anaerobic species. In the course of their study of *Bacillus bifermentans*, Clark and Hall (47) discovered positive but "weak" cross-agglutination between *B. bifermentans* and *B. sordellii*. Moreover, the serum of rabbits immunized with *B. bifermentans* was found to be protective to guinea pigs injected with toxin of *B. sordellii*. However, since the pathogenicity of *B. sordellii* contrasts sharply with the non-pathogenicity of *B. bifermentans*, Clark and Hall did not recommend combining these species. Spray (304) has also respected the difference in pathogenicity and has made it the only point of separation in his key to the spore-forming anaerobes. The relationship has been further investigated by Stewart (310), who has found 1 cc. of an experimental antiserum for *C. bifermentans* to protect mice against 2 to 5 M.L.D. of the toxin of *C. sordellii*. Positive agglutination and precipitation reactions have also been obtained, thus linking the two species. Because they are also indistinguishable in

morphology, colony formation, and biochemical reactions, consolidation has been recommended under the name: *C. bifermentans*, which has priority over *C. sordellii*. The antitoxin would then be called by Stewart bifermentans antitoxin, but whether this terminology will come into general use remains to be seen. A somewhat analogous case involving *Bacillus oedematis maligni* II of Novy (now called *Clostridium novyi*) and *C. oedematiens* will be discussed later.

Ictero-hemoglobinuria of cattle in Nevada is caused by an organism which was at first called *Bacillus hemolyticus bovis* and later, *B. hemolyticus* (331, 333). For a time, there was some question whether it also could be identified with *C. oedematoides*, but apparently the latter is merely associated with it in certain cases of the disease (120).

The toxins of Clostridium parabotulinum and Clostridium botulinum[3]

After recognition by Leuchs (182) of the production of multiple toxins by *Clostridium botulinum*, Burke (45) reported two distinct toxin groups, arbitrarily called Types A and B, in the 12 American strains studied by her. The antitoxin of one type neutralizes the toxin of the homologous but not of the heterologous type. An important contribution by Bengtson (16, 18) revealed a third toxin group, Type C, represented by cultures[4] from larvae of *Lucilia caesar* taken from a chicken, which had

[3] The nomenclature of the botulinus-parabotulinus group is particularly confusing, because of a lack of understanding of their separation on the basis of proteolysis. The terminology was introduced by Bengtson in 1924 (18). As recently as 1937, however, the distinction between *C. botulinum* and *C. parabotulinum* was not appreciated, which unfortunately adds to the confusion (369). It is beyond the scope of this review to argue the nomenclature; the original basis of separation and discussions regarding it may be found in the following articles: 18; 112; 131; 186; 221–224; 268; 288; 315–319; 321, pp. 687–690, 1276–1279; 350, p. 107; 358, pp. 332–340; 369. We have accepted the terminology of Bengtson and have therefore used *C. botulinum* to refer to non-proteolytic cultures found within the toxin types B, C, D, and E. Proteolytic cultures, termed *C. parabotulinum*, have been found thus far only within the toxin types A and B.

[4] The Type C organism has been called *Clostridium luciliae* by Bergey (29) and by Spray (304), but the name has never come into common use and seems not likely to, because it is at variance with the Bengtson nomenclature.

died of limberneck. Probably identical is the organism later isolated by Graham and Boughton (106), and in the same toxin group, at least, is the organism of Seddon (287, 288) from botulism of cattle in Tasmania. Pfenninger (248) showed that the Seddon organism produces a toxin, which can be neutralized by the Type C antitoxin prepared by Bengtson, but that the antitoxin for the Seddon culture neutralizes only its homologous toxin. These observations have been confirmed, and the two factors have been designated C_α and C_β, within Type C toxin (110, 112, 114). A Type D toxin was added after study of the organism associated with botulism or lamsiekte of cattle in South Africa (224, 315, 316, 319). Finally, a Type E was proposed by Gunnison, Cummings and Meyer (111) to designate the toxin of an organism obtained from Russia and originally isolated from spoiled fish. (Topley and Wilson (321, p. 689) have erroneously cited Type E as the cause of equine botulism in South Africa; in reality (268), the disease is due to *C. botulinum*, Type C.) Apparently the organism of Hazen, which was unfortunately incompletely reported (132), is also a Type E culture.[5] Thus, there are now five types of the botulinus toxin.

The work of Mason and Robinson (216) is of interest because of its extensive experiments on the antigenic components of these toxins. The A and B toxins appear to be monospecific, whereas the C and D toxins are not so simply constituted. It has been claimed that C toxin is composed of two major fractions, C_1 and C_2, which may vary quantitatively with the conditions of incubation; and that D toxin is present, but in small amount only, in the so-called Type C toxin. Although the Type D organism produces chiefly D toxin, there is also apparently some admixture of C toxin. Confirmation of this distribution of the fractions is needed; and extended study should be made of all available cultures of these types, with particular attention to the conditions for production of the toxins. The new Type E strains and others, which may be isolated from widely separated geographical regions, should be included in the study.

[5] Personal communication from Dr. K. F. Meyer. A paper reporting this finding is in preparation.

The toxins of the Clostridium welchii group

From the time of isolation of the organism now called *Clostridium welchii* (*Bacillus aerogenes capsulatus, B. phlegmonis emphysematosae, B. perfringens*) there have been many reports of its remarkable pathogenic action. The acid (principally the butyric acid) was at first regarded as a tissue irritant and debilitant (49). As late as 1916, it was argued that no true toxin was concerned but that the acid injury was a sufficient cause of the lesions of gas gangrene (307). The mechanical effect of the gas in blocking circulation and contributing to the spread of infection was also considered important (202). The severe toxemia and the rapidly fatal outcome, however, made it seem likely that a potent toxin was involved. In 1917, Bull and Pritchett (42–44) obtained the soluble toxin *in vitro* and prepared the antitoxin. The main properties of the toxin were soon determined; and again a multiplicity of toxin elements was indicated. These have been named after their apparent physiological action: a hemotoxin or hemolysin (42, 46, 88, 142, 157, 242, 280, 357, 374); a myotoxin (143); a neurotoxin (346); another toxin said to act specifically on the blood vessels (347); "necrotic and lethal" toxin (99); the "acute lethal" toxin of Shiraishi (289); and finally the so-called "pseudotoxin" (7, 170), which is heat-stable, non-antigenic, and non-neutralizable by antitoxin. This "pseudotoxin" may be the histamine or histamine-like substances found among the growth-products of *C. welchii* (162). With such an array of toxin elements (though some of them appear to have been proposed on very meagre evidence), it is no wonder that the testing of toxin and antitoxin of *C. welchii* presents considerable difficulty. The proportion of hemolysin, for example, varies widely (357), and this variation interferes with the measurement of the protective power of an antiserum by the titration of its antihemolysin content (99, 143, 215, 252, 253, 354).

Further evidence of the complexity of *C. welchii* toxins appears, when one considers the several organisms resembling, but apparently not identical with, the *C. welchii* of classical gas gangrene. In recent years many reports have claimed, or questioned, the association of *C. welchii* with intestinal disorders of man, such as

flatulent diarrhea (161, 234, 311), or the toxemia of intestinal obstruction (146, 201, 233, 320, 366). Also, in diseases of the enterotoxemic type among domestic animals, there are found organisms resembling *C. welchii*. Lamb dysentery in Great Britain, one of the first of the enterotoxemias to be investigated, is ascribed to the lamb dysentery bacillus of Dalling (62, 92), sometimes called *Bacillus agni*. The bacillus is not identified as *C. welchii* because of minor physiological differences, and because the antitoxin for *C. welchii* is not capable of neutralizing the toxin of the Dalling bacillus, although its antitoxin is capable of neutralizing the toxin of *C. welchii*. However, the hemolysins of the two organisms can be neutralized by either antitoxin. Soon another organism from a sheep disease, called *struck* in England, was discovered by McEwen (195–198). It resembles both *C. welchii* and the Dalling bacillus, but differs in a few physiological reactions, and significantly in its toxin-antitoxin specificity. The toxin of McEwen's organism, *Bacillus paludis*, is not neutralized by the antitoxin of *C. welchii* (human), nor the toxin of *C. welchii* by the antitoxin of *B. paludis*. McEwen inferred, therefore that his organism differed also from Dalling's bacillus, especially since he was not at first able to show that *B. paludis* produces a hemotoxin. Complicating still further this group of *welchii*-like organisms of animal diseases is the organism found in enterotoxemia of sheep in West Australia (25, 27) and the "pulpy kidney" disease of sheep reported from New Zealand (97, 98), Tasmania (26, 244), Australia (26), Palestine (96), and North Wales (229, 230). Apparently the same organism occurs in other herbivorous animals, and it has been considered as the possible etiologic agent in the grass disease of horses (105). Bennetts (25) discovered and described the organism of sheep enterotoxemia in 1932. Again, because of the differential specificity of its toxin, as evidenced in cross-protection experiments with the classical *C. welchii*, *B. paludis* of McEwen, and the Dalling bacillus, Bennetts considered his organism a new species, which he named *Bacillus ovitoxicus*.

Prophylaxis of the various sheep diseases has been tried with both antitoxin and toxoid vaccinations (27, 68, 69, 212). In

experiments of this nature, it is important that the cross-protective powers of the antisera produced for the several species be known, because investigation of the distribution of the diseases has shown that the organisms may occur widely and at random, —*B. paludis* has been reported in France, Greece and Turkey; *B. ovitoxicus* in Scotland, North Wales, Palestine, United States, etc. (25, 27, 64, 96, 231, 355, 356). Studies of the toxin-antitoxin relations were made at the Cambridge Institute of Animal Pathology and the Wellcome Physiological Research Laboratories at nearly the same time. The Wilsdon report from Cambridge was published first, showing clearly four organisms, Types A, B, C, and D, on the basis of toxin-antitoxin reactions (367). Type D was then new in England and, although subsequently shown to be *B. ovitoxicus* Bennetts (368), it is still often called the Wilsdon Type D organism. The toxins and antitoxins of these organisms display considerable overlapping of factors, the details of which as determined by cross-protection experiments are as follows:

ORGANISM	TOXIN TYPE	TOXIN FACTORS	ANTITOXIN NEUTRALIZES
Cl. welchii	A	W	A
Dalling's	B	WXZ	A, B, C, and D
B. paludis	C	WZ	A, B, and C
B. ovitoxicus	D	WX	A and D

Wilsdon closes his report with the statement that "although the strains can also be grouped according to one or another of their biochemical characters, it is held that these are of minor importance compared with differences in antigenic structure revealed by toxin-antitoxin reactions."

In 1933, Glenny, Barr, Llewellyn-Jones, Dalling and Ross (100) of the Wellcome Laboratories announced the toxin fractions α, β, γ, and δ; to these was added the factor ϵ, said to be present in Dalling's early cultures of the lamb dysentery bacillus but weak or lacking in many Type B strains of the present day (211). Prigge (252–254) has recently added a ζ factor, so far

known only in the true *C. welchii* or Type A. The properties of these factors in physiological terms are (65, 211, 252):

α—hemolytic, lethal and necrotic
β—lethal and necrotic
γ—lethal
δ—hemolytic
ϵ—lethal and necrotic
ζ—lethal

The distribution of these factors in the respective toxin types is as follows (65):

TOXIN TYPE	FACTORS					
	α	β	γ	δ	ϵ	ζ
A	+	−	−	−	−	+
B	+	+	+	±	+	
C	+	+	+	+	−	
D	+	−	−	−	+	

With six factors involved in the toxin complex, instead of the three (WXZ) originally recognized by Wilsdon, problems of cross-protection for the four types of *C. welchii* organisms will require reinvestigation, the more so since five of the six factors are reported to be lethal. Nicholson (235) studied the action of the whole toxins of the Type A, B, C, and D organisms on the circulatory and respiratory systems; but his findings are difficult to interpret for obvious reasons.

The most remarkable organism of the four is Type B, or Dalling's lamb dysentery bacillus, which has at least five of the toxin factors. Its antiserum should therefore protect against the toxins of Types B, C, and D. The discrepancy to be explained is that it also protects against Type A toxin, according to Wilsdon and Montgomerie and Rowlands (232, 367), although the lethal ζ toxin of Prigge is not known to be formed by Dalling's bacillus. Similarly Type C antiserum, which should lack ϵ antitoxin, protects against Type B toxin, which has the ϵ factor. That there is still some fine point of relationship to be worked out between

these two types is probable, because Dalling's laboratory has record of a Type B culture, which has become Type C by loss of the ϵ factor (64). The classical *C. welchii* would seem to be the most restricted of all in respect to toxin factors, yet there is still something to be explained in the cross-protective power of its antiserum. Commercial antisera for *C. welchii* are of Type A mono-specificity, as judged by their neutralizing power. Nevertheless, they will protect guinea pigs from infection by Type D organisms, which differ from the Type A organisms in possessing the ϵ toxin factor (31, 32).

The suggestion has been made (36) that gangrene antiserum for man might be improved by being made polyvalent, for apparently there is ample opportunity for infection of man by any member of the *C. welchii* group. Thus far, however, Types B, C, and D are known as pathogens of domestic animals only; but they can upon parenteral injection give rise to typical gas gangrene in experimental animals (197a). Very little is known of the distribution of the types of *C. welchii* in the intestinal tracts of man and other animals. Borthwick and Gray (35) have evidence of only Type A in human feces, whether of normal individuals or of a patient with pernicious anemia. In rabbits only Type A has been found and in guinea pigs only Type D (34). In the dog both Types A and D were found, but investigation showed that the newly-isolated Type D strains tended to revert to Type A, which involves only loss of their X factor (Wilsdon's terminology). A further study of the D→A conversion would be of decided interest, in view of Borthwick's finding (31) that commercial mono-A type sera will protect experimental animals from either Type A or D infections, although neutralizing only Type A toxin. Prigge's last contribution (254) on the importance of the ζ toxin of Type A organisms in relation to the protective power of the sera, should be considered also in the problem of the relation between Types A and D organisms. For the maximum protective power of *C. welchii* antisera, Weinberg (341, 352, 353) has advocated those sera which are both antitoxic and antibacterial. Such sera he would call "holosera" or "anti-

exo-endo-toxic sera." Their advantage has not yet been adequately shown.

Other papers have appeared characterizing the interrelations of these organisms and have confirmed the main facts (31, 72, 122, 211, 217). In one case, cultures derived from single cells were used to dispose of the possibility that the overlapping of toxins and certain irregular proteolytic action of the types might be due to mixture of the closely related organisms (210).

Such is the present state of knowledge of the toxins of the *C. welchii* group. Some points of uncertainty remain: whether the lamb dysentery strain of 1923 possessed the toxin factor for *B. ovitoxicus*, as its serum seemed to indicate (63); why there are discrepancies in hemolysin production by cultures tested at different times (195, 252, 354); how stable the toxin types are, in view of the D→A conversion, and so on. The etiologic agents of sheep diseases in different parts of the world are still being investigated. Recently, for example, the "bloedpens" of South Africa has been identified with the lamb dysentery of Great Britain, and its causal organism also found to be *C. welchii* Type B, complete with the ϵ toxin factor (211, 213). Lamb dysentery in Montana is apparently not the same (208). Enterotoxemic jaundice or sheep yellows in Australia is due to *C. welchii* Type A (269). The peculiarities of the enterotoxemias are not yet fully understood. There is an intestinal factor, possibly trypsin, obtained in filtrates of the intestinal contents, which when added to Type D toxin renders the mixture very much more toxic to the mouse (37, 38). A "diffusion factor" in cultures of various anaerobes, including *C. welchii*, has also been reported (184). Until more is known of these factors which apparently affect the permeability of the intestinal walls, it is difficult to evaluate the reports. They are perhaps important in determining the pathogenicity of the *C. welchii* regularly found in the intestinal tract.

Short reviews of the interrelations of the toxins and of the etiology of the various diseases due to the *C. welchii* group are available (64, 72, 373). Discussions at scientific meetings have brought out interesting points, some of which have not been made in other published papers (32, 36, 65–67, 286, 342, 343).

The toxin of Clostridium oedematiens and its near relatives

Clostridium oedematiens was found by Weinberg and Séguin in certain cases of gas gangrene, occasionally alone but more often associated with *C. welchii*. Its toxicity was proven and it was successfully differentiated from *C. septicum* and *C. welchii* (359, 363). The therapeutic value of the *C. oedematiens* antitoxin has justified its inclusion in the polyvalent sera for gas gangrene (94, 204). Fortunately, the toxin is monotypic, for Weinberg, Nativelle, and Prévot (358) have stated that the antisera will neutralize toxin of all strains, whether of human or animal origin. Toxigenicity is relatively weak, or appears to be so because of the instability of the toxin. The properties of the toxin and the conditions for its preservation have recently been investigated (23, 339).

There remains, however, the question of separation of the toxin of *C. oedematiens* from those of certain of its close relatives. Sacquépée (275, 277) discovered an organism at first called "bacille de l'oedème malin," later *Bacillus bellonensis*. There was for a time doubt of its purity and of its relation to *C. oedematiens* (274, 276, 361), but it has recently been accepted by Weinberg (358) as a separate species with a specific toxin. Sacquépée (277) distinguished it from *C. septicum* by toxin-antitoxin reactions.

Another organism very close to *C. oedematiens* is the *Bacillus oedematis maligni* II of Novy (now called *Clostridium novyi*). It is often considered synonymous, but Weinberg regards it as an atoxic variety of his organism, remarking that it would probably have been so recognized had it been discovered *after*, instead of *before*, *C. oedematiens* (358).

Bacillus gigas Zeissler and Rassfeld (380) is also similar to *C. oedematiens*. It was found in a disease of sheep, resembling bradsot or braxy, and was considered different from *C. oedematiens* principally because of the conspicuous size of its cells (1 to 2 by 4 to 20 micra) and because of certain cultural differences. It produces a toxin, whose relation to the toxin of *C. oedematiens* has apparently not been explored. Kraneveld, who found *B. gigas* in diseased animals in the Dutch East Indies, also reported

another organism (172), the bacillus of *osteomyelitis bacillosa bubalorum* of the Indian water buffalo; he later identified his organism with the "Novy group" as defined by Miessner, Meyn, and Schoop (173, 227). None of the descriptions of *B. gigas* is as complete as one would wish, and further study of its toxin is needed. The same may be said of other oedematiens-like organisms, which have been found in animal diseases: bradsot and braxy, infectious necrotic hepatitis, black disease, and big head of rams. These and probably others of the so-called braxy group are not as yet well characterized (with a few notable exceptions, *e.g.*, black disease (243, 328)). Perhaps in time a complexity of causal organisms like that of the *C. welchii* group will be found, and if so, it is not improbable that knowledge of the toxin specificities of the group will aid in its resolution.

The toxin of Clostridium chauvoei

Clostridium chauvoei should perhaps be considered above, close to *C. septicum*, for it is of that general affinity. We choose to discuss it here, however, because for a time there was debate as to whether it formed a toxin, and certainly there are strains which have not been shown to do so in the laboratory. It thus represents, in a sense, a transitional organism to the non-toxigenic forms, and will serve to introduce mention of "non-toxic or atoxic" strains of supposedly toxigenic species.

C. chauvoei is the cause of blackleg, symptomatic anthrax, quarter evil, or "Rauschbrand," as the disease is called in different localities. Production of toxin was claimed by several early workers (151, 159, 171, 179, 236, 238) but denied by Scott and others as late as 1928 (130, 283–285). Recent work, however, has shown that toxin can be elaborated under proper conditions of culture (165, 214). The explanation of the disagreement seems to be that toxigenicity is easily lost, and consequently non-toxic strains are more generally encountered in this species than in other common anaerobes (8, 66, 130, 151, 167, 179). Perhaps because of the instability of the toxigenicity, more than usual effort has been made to practice active immunization against this disease. Many types of blackleg prophylactic products have

been tried: filtrates of cultures (at times probably toxin-containing), vaccines of various kinds, anacultures, even infected muscle powder and spore vaccines. These products may carry also lysins; and the immunity which they induce is undoubtedly complex. Hence it is very difficult to assess either the importance of the antitoxic factor in the immunity produced, or to claim for it any conspicuous laboratory usefulness in diagnosis or typing. As will be seen below, agglutination, precipitation, and complement fixation may be resorted to in the classification of such organisms.

The problem of atoxic strains

It must be emphasized in passing that *C. chauvoei* is not unique in possessing atoxic strains. The literature cites the following: *C. tetani* (53, 84–86, 89, 163, 164, 206, 262, 263, 326); *C. welchii* (34, 35, 144, 183, 199, 201, 239, 240, 262, 289, 291, 336); *C. botulinum* and *C. parabotulinum* (17, 18, 45, 76, 113, 216, 241, 248, 267, 268, 322, 334); *C. oedematiens* (23, 39, 48, 359); *C. hemolyticum* (332); and the bacillus II of Ghon and Sachs (314). In most of the above cases the atoxic strains were found at isolation, or they developed spontaneously during laboratory cultivation of the originally toxic cultures. Occasionally, cultivation to produce variants was intentionally undertaken.

Loss of toxigenicity often throws a variant extremely close to the borders of another species. Who is to say that an atoxic strain of *C. tetani* is not within the confines of *Clostridium tetanomorphum*, or an atoxic *C. welchii* within those of *C. butyricum*? This weakness of the method, or perhaps one should say of our conceptions of the species, can in some cases be corrected by correlation of other antigenic reactions, agglutination or precipitation or complement fixation. Reference will be made to the placing of atoxic strains of *C. tetani* and *C. parabotulinum* by such means. But at least the possibility of missing entirely the relations of a strain, because of its atoxicity, brings the realization that the toxin neutralization test is not even a generally applicable method for typing anaerobes, since the many nontoxic species are entirely beyond its reach. In general applica-

tion therefore, any method of analysis of the intact cellular antigens is to be preferred. In our opinion, agglutination, properly carried out, serves that purpose, and its usefulness will be discussed next.

THE AGGLUTINATION REACTION APPLIED TO SPORE-FORMING ANAEROBES

First recognized during a comparison of the blood of animals normal and immune to tetanus, agglutinins for anaerobes were found soon after the phenomenon of bacterial agglutination itself was known (56, 57, 273). Early application was made to the distinction of two anaerobic species, *C. chauvoei* and *C. septicum*, by Leclainche and Vallée (178). Since then the method has had extensive use, as we can testify, having read more than 250 papers on this one phase of the serology of anaerobes! There has not been complete satisfaction with the method; and it will perhaps best serve the present purpose to mention some of its difficulties and failures, as well as its usefulness.

The first hint of difficulty appeared in a paper by Nicolle and Trenel (237) on the variability of agglutinative aptitude and agglutinogenic function, in which it was stated that anaerobes are: "infiniment moins sensibles et. . . . souvent variables." Nevertheless, in the monographs on anaerobes after the World War (52, 75, 350, 378, 379) it was possible to list those organisms for which satisfactory agglutinating sera had been prepared, and the few for which they had not.

Although Meyer (200) had in 1915 recommended agglutination, Zeissler (376), Heller (135), and Hall (119) categorically condemned it as an aid in the typing of anaerobes. Hall and Stark (128) from their experience with *C. sporogenes*, however, stated that "one should regard serological agglutination as a satisfactory criterion of species identity in properly controlled positive tests, but not of differentiation in negative tests." One of the chief complaints concerned the instability or "auto-agglutinability" of the anaerobic cell suspensions used as antigens; for instance, "L'auto-agglutination des cultures du *B. oedematiens* en milieux liquides est une des caractéristiques de cet anaérobie. Il est

donc impossible d'étudier l'agglutination de ce germe en recourant aux procédés ordinaires." (345). It is unfortunate that the *Manual of Methods for Pure Culture Study of Bacteria* (41, 121) still recommends the use of broth cultures of anaerobes directly as antigen, either for injection or for macroscopic tube agglutination. Particularly in glucose broth, and some form of it is likely to be used with anaerobes, the acidity resulting from fermentation may cause flocculation of the cells. Heller (135) had the key to correction of this difficulty, when she secured stability by adjustment of the electrolyte concentration and the pH of the antigen suspensions. Snyder (294) recently re-emphasized the danger of non-specific agglutination by acid, and established a critical pH for the species with which he worked. If broth cultures are ever to be considered as antigens, it is important to know that the cells in broth may be flocculated at a pH distinctly higher than in distilled water. To avoid the errors due to autoagglutination, Plaut (249) devised a dark-field method for agglutination in hanging drops; he claims that true agglutination is easily detected by the loss of motility of the clumps.

Two other complaints, (a) that for certain groups of anaerobes it is very difficult or "impossible" to produce agglutinating sera, and (b) that for others the agglutination may be ultra-specific, can best be dealt with by presenting the pertinent data for the major groups of anaerobes.

But first we would point out that methods of agglutination have undergone considerable change from the first simple mixing of culture and serum. The Oxford method of achieving "standard agglutination" was a distinct advance. It was followed by the flagellar-somatic agglutination of motile organisms, evidence for at least a dual nature of the agglutinogens. This concept was shrewdly criticized by Tulloch (327), as it was then a purely qualitative theory, little more than the old mosaic hypothesis of Durham and Ehrlich. But there can now be no doubt that separate antigens do exist independently within the cell, as evidenced by the many isolations of protein and polysaccharide fractions. Furthermore, the apparent success in resolving the *Salmonella* group by analysis of their somatic and flagellar anti-

gens is an outstanding example. The "Salmonella Subcommittee of the Nomenclature Committee of the International Society of Microbiology" (279) has recently published its findings on the genus *Salmonella*, in a scheme based on the Kaufmann-White antigenic factors of the group. It is of interest also that the League of Nations Health Organization has in April, 1937, adopted a detailed technique to be used in the agglutinative diagnosis of enteric infections (82). These recent uses, and a review and interpretation of the techniques of agglutination by Cruickshank (58), are commended to the reader. For specific agglutination methods which have succeeded with anaerobes, the following papers may be consulted: 83, 109, 136, 190, 281.

The agglutination of Clostridium tetani

As mentioned above, *C. tetani* was the first of the anaerobes to reveal its agglutinative powers. However, nothing of importance concerning the typing of the species appeared until Tulloch (324–326) discovered a subdivision into five serologic types. On a combination of the evidence from several workers (14, 50, 51, 85) nine types are now known. In 1928, the flagellar-somatic method of agglutination was applied to *C. tetani* by Felix and Robertson (83), and the dual nature of the agglutinogens was found for the first time to be true for a motile anaerobe as well as for motile aerobes. The type-specificity of former groupings (only seven were then known) was shown to lie within the reactions of the flagellar or thermolabile H antigen. There was included in the study a so-called "pure O" strain,—a non-motile variant by chance devoid of the H antigen proper to its species. Parenthetically, it may be said that such strains, if available, are used to produce potent somatic antisera; otherwise, the H antigen of a motile strain may be destroyed by heat or chemicals and the resulting product used as the O antigen. Antiserum for the pure O strain of *C. tetani* was found to agglutinate in the "small flaking manner"; and from this it was concluded that "in the stable antigen the group relationship is very close indeed," in contrast to the type-specificity of the H antigen. With so-called "smooth" and "rough" strains of *C. tetani*, Condrea (53)

soon confirmed the existence of H and O antigens in the species. His smooth strains were complete with both antigens, whereas the rough possessed only the O. The O antigen of any given rough strain, however, was like that of the smooth strains of its group. In other words, overlapping or very close relationship of O antigens between the groups was not confirmed. In fact, Condrea stated clearly that in his opinion such cross-reactions must be due to admixture of types! There the matter rested until Gunnison (109) recently presented a thorough study of 67 strains, representing all nine of the types now known. As a result of the cross-testing of all strains with the antisera for all nine groups, "there was no differentiation among the 67 strains tested." Absorption tests being confirmatory, it was concluded that there is a common O antigen with no type specificity. But in addition to this common O antigen, absorption tests revealed another O antigen, which divides the collection into two subgroups. Types II, IV, V, and IX possess this second O antigen, whereas I, III, VI, VII, and VIII lack it. The complexity of agglutination of *C. tetani* is therefore still considerable, but it has been clarified; and it should now be possible to identify an unknown strain as belonging to the species, *if* it possesses the common O or species-specific factor. *Clostridium tetanomorphum* was tested by Gunnison and, although it gave a group reaction to 1:640 titre, absorption with it did not lower the original O titre of the serum for *C. tetani*, thereby proving that *C. tetanomorphum* has not the species-specific O factor of *C. tetani*. *Clostridium tertium*, *Clostridium putrificum*, and *Clostridium sphenoides* were found to be even further removed in group relation. Among the 67 strains of *C. tetani* tested 11 were atoxic and therefore could not have been recognized as belonging to the species by a toxin-antitoxin test.

The agglutination of Clostridium septicum

Some early studies on the agglutination of *C. septicum* were made to distinguish it from *C. chauvoei* (178, 194, 283), apparently with success; although recently "a close relation" between the two organisms has been indicated by a combination of evi-

dence from their agglutination and complement fixation reactions (349). The complexity of its agglutination first became apparent in the report by Robertson (264) which indicated three subtypes in the species. Later, when flagellar-somatic agglutination was being applied to the species by Felix and Robertson (83), the same sera were reinvestigated, although nearly nine years old. It was found that the original subdivision depended upon the H antigen reactions, in this species as in *C. tetani*. Four groups were finally established by Felix and Robertson. In the somatic sera, they found the cross-reaction of the O antigens of the several groups to indicate close group relationship, but not identity. In 1928 Davesne (71) mentioned six subgroups in direct agglutination and therefore presumably dependent upon H antigens, but Bengtson (19) in 1933 still recognized only "at least four" groups.

The somatic or O antigen of *C. septicum* has an added importance in relation to protective immune sera, and has therefore been investigated by several workers. Robertson and Felix (266) claimed definite, but type-specific, protective value of the O immune serum. Weinberg, Davesne and Haber (348) were unable to confirm its value, and since antitoxic sera were successful, they saw no reason to change therapeutic procedure. Henderson, (139, 140), continuing investigation of the O antigen both for its species relationship and for its possible prophylactic value, confirmed the Robertson-Felix report. He did, however, clarify their statement concerning the close relation of the O antigens of the several groups. There is an identical O antigen in Groups II and IV; and "considerable overlapping," but not identity, in I and III. In conclusion, he suggested that the O antigen relationship be made the primary basis for grouping within the species, and that the H antigen relations be considered secondary. This suggestion has not been widely recognized, but it is, in fact, a good one, as the O antigen is the more stable. Henderson's most recent contribution (141) suggests that in addition to the O antigen, there is a heat-labile antigen, possibly identical with the H antigen, which has importance in antibacterial sera.

The agglutination of Clostridium chauvoei

After the early separation of *C. chauvoei* from *C. septicum*, the agglutination of *C. chauvoei* itself was examined (156, 226, 377, 378, 381). Strong reactions with "no serological differentiation of races" were found to occur, regardless of the ovine or bovine origin of the strains. Nevertheless, controversy as to the identity of so-called "spontaneous Rauschbrand" of sheep *versus* the "wound Rauschbrand" of cattle (209, 225, 226, 370) was not finally settled until a full antigenic analysis of the species by flagellar-somatic agglutination was accomplished. On this basis the H antigen was found by Roberts (260) to be different in the ovine and bovine strains, whereas the O was identical. Henderson (136), repeating the study, agreed upon the uniformity of the O factor; in fact, he claimed exceptional importance for it in view of the subdivision of species by means of the O factor in the case of *C. septicum*. He did not, however, confirm the separate entity of the ovine and bovine H factors. Instead he found an equal distribution of the H antigen in all but two of his strains (both English ovine which had a common H factor different from that of the other strains). A minor component of the H factor is shared by all strains. Later work deals only with the immunizing value of the O antigen in vaccine (137, 261) or in antibacterial serum (138).

The agglutination of Clostridium parabotulinum and Clostridium botulinum

The first investigators to study extensively the agglutination of *C. parabotulinum* (then called *B. botulinus*) were Starin and Dack (305) and Schoenholz and Meyer (281). The latter, examining 111 strains of diverse origins, found that the Type A strains could be divided into three or four subgroups and the Type B strains into at least two subgroups by agglutination and absorption tests. In each toxin type one group was left a heterogeneous collection of ultra-specific and unclassified strains. Only between certain groups was cross-agglutination noticed. Generally, negative results were obtained on cross-tests of the botulinus and the non-toxin-producing anaerobes, such as *C. sporogenes* and *C. bifermentans*.

Using the flagellar-somatic agglutination technique in an extensive re-study of over 160 cultures of the proteolytic *C. parabotulinum*, including the original collection of Schoenholz and Meyer, McClung (186) showed that the subgroups reported by previous workers were based on the reaction of the heat-labile antigens. Without exception, strains of *C. parabotulinum* Types A and B reacted with a serum produced against the heat-treated antigen of any strain regardless of toxin type. Mirror absorption experiments confirmed the identity of the heat-stable somatic antigen. Townsend (322) and Gunnison and Meyer (113) had previously classified various non-toxic strains by their reaction to titre with specific antisera; these strains also reacted with the somatic antisera and could not be distinguished from the toxic cultures. Even more interesting is the possibility of using a somatic antiserum as a specific reagent for the proteolytic *C. parabotulinum*, as revealed by the cross-tests of these organisms and various related species, in particular *C. sporogenes*. Although some cross-reaction is evident prior to absorption, it appears that somatic antiserum for *C. parabotulinum*, preabsorbed by *C. sporogenes*, may give a reaction with the homologous species only. However, the strains of *C. sporogenes* do not all react uniformly; therefore a decision as to the group relationship of this species to *C. parabotulinum* must await further examination. Nevertheless, the great importance of such a serum is evident, and it is hoped that the future will soon produce a fuller study.

The heat-stable antigens of a small number of strains of Types B, C and D of *C. botulinum* were included in McClung's study (186), and their apparent specificity was noted. Prior to this, the agglutination of Types C and D had been examined by several workers (110, 224, 248, 268), and they too had found no cross-reaction with the type sera of *C. parabotulinum*.

The agglutination of Clostridium welchii

It is not yet possible to present the agglutination of *C. welchii* in anything like the state of agreement that has been reached for *C. tetani* and *C. parabotulinum*. There is nothing but confusion at present, and apparently for two reasons. As late as 1929,

Robertson (265) wrote that "there is no evidence that agglutinins have been produced in the bodies of animals injected with *B. welchii.*" It is true that the literature contains rather numerous statements that such agglutinins are not, or at least not readily, produced. That they can be formed, however, is shown in some of the early studies. Several recent reports (149, 259, 344, 367, 375) of even comparatively high titres have removed all doubt on this score. However, the second difficulty, namely, that the antisera are conspicuously strain-specific, has not been overcome. We can do no more than quote Wilsdon (367): "While it is possible to prepare agglutinating sera in the case of a number of strains of *B. welchii*, there is little likelihood of formulating a satisfactory classification of the members of that group on the basis of their agglutination reactions." It is only fair to point out, however, that he refers to the entire *C. welchii* group, human and animal. It must also be pointed out that *C. welchii* has not been fully analyzed for antigenic structure by the newer techniques. The organism is non-motile, an exception to most of the group of spore-forming anaerobes. For this reason it has no flagellar antigen; but it does have capsular antigens to be considered. The precipitin and complement fixation reactions, which incidentally have not been adequately tried for the species, should offer possibilities.

The agglutination of some proteolytic species

It is convenient to consider together the agglutination of a group of non-pathogenic proteolytic anaerobes. There is as yet no certainty of a subdivision into types within these species, but there is interrelation of certain species, as recently revealed by agglutination studies.

Clostridium sporogenes is probably poorly defined, in the sense that it is considered a widely distributed species and that non-pathogenic proteolytic forms are often assigned to it without detailed study. Perhaps then confusion is to be expected. The several reports (75, 128, 200, 246, 378, 379) are not in agreement, except upon the fact that not all strains of *C. sporogenes* agglutinate in all antisera. Three groups were recognized by Zeissler and

Rassfeld (379), but Hall and Stark (128) claimed agglutination, if only to low titre, with all strains. Lack of homogeneity is again indicated in the two recent reports upon cross-agglutination of *C. sporogenes* and certain other proteolytic species. McClung (186) noted irregularity of behavior of certain strains of *C. sporogenes* in absorption of the antisera for *C. parabotulinum*, as discussed above. Another example of the interrelation of "certain strains" of *C. sporogenes* with another anaerobic species is reported by Smith (292). Stable rough variants of *C. histolyticum* are said to react, though not to full titre, in an antiserum for *C. sporogenes* (strain 319 only); a reciprocal reaction was also obtained with this strain. Other strains of *C. sporogenes* reacted only to 1:20 or 1:80 dilution of antisera for the rough variants of *C. histolyticum*. These findings were offered in support of a claim (147) that rough variants of *C. histolyticum*, which resemble *C. sporogenes* in colony form and certain biochemical properties, are not chance contaminants but show "genetic relation" to *C. sporogenes*. Smith, however, does not claim direct identity of the species, but merely "a number of antigens common to both *Clostridium sporogenes* and *Clostridium histolyticum*." Further study will be necessary to establish these relations.

Clostridium bifermentans has been studied recently in order to clarify its relation to the so-called *Bacillus centrosporogenes*. Since one of the principal differences claimed was the absence of motility in *C. bifermentans* and its presence in *B. centrosporogenes*, a study of the flagellar and somatic antigens seemed indicated. Proof of the complete identity of the antigens, coupled with the previously known identity of other characters makes it no longer necessary to recognize two species. Because *C. bifermentans* has priority, its name has been retained (47, 192). The merging of another species, *C. sordellii*, with *C. bifermentans* has recently been proposed, partly on the basis of cross-agglutination. Details of this proposal by Stewart (310) have been discussed under the section on the toxin of *C. oedematoides* (syn. *C. sordellii*).

Several other putrefactive species have been studied in recent years by Hall and his associates, and in each case agglutination has aided in establishing the species: *Bacillus paraputrificus*

Bienstock and *Bacillus capitovalis* Snyder and Hall (295, 297); *Bacillus difficilis* Hall and O'Toole, in which subgroups were recognized by Snyder (296); *Clostridium fallax* and *Clostridium carnis* (77); *Bacillus bifermentans* as separate from several others (47); and *Bacillus paraputrificus* Bienstock and "*Bacillus innutritus*" Kleinschmidt (124).

The agglutination of the saccharolytic anaerobes of the butyric group

Butyric anaerobes of the soil are a heterogeneous lot, and no one knows how many species to recognize! Serology has not given a complete answer by any means, but it has, we believe, contributed to the definition of several species (188, 190, 191). One such is *Clostridium acetobutylicum*, the organism of the original Weizmann method of butyl alcohol fermentation. An unusually complete set of cultures was available for the serological study of the species: two representatives of the original Weizmann isolation (one untouched for 17 years) and twenty other cultures isolated by various workers over a period of 20 years. A complete antigenic analysis was made by the H and O technique by both direct agglutination and absorption (190). Unquestionably, every strain belonged to the species, and there was no need to subdivide the group into types on the basis of either flagellar or somatic agglutination. The only variation encountered was quantitative, in the sense of Schütze (282); namely, certain strains were *master strains* with full complement of H and O factors, and others were *substrains* with the same antigens, but with some deficiency of the H factor. Since the latter is the flagellar antigen, it is not surprising that quantitative variation should occur from time to time.

In the group reaction of *C. acetobutylicum* with other butyric anaerobes there is nearest affinity to the retting organism, *Clostridium felsineum* Carbone, and to a new pigmented anaerobe which appeared different and which was later proposed as a new species, *Clostridium roseum* McCoy and McClung (191). The interrelations of the three species were analyzed with the following results (188). A common somatic O factor occurs in the three organisms, but each is distinguished by an H factor, which

is dominant and species-specific. The group reaction in the *C. felsineum: C roseum* and *C. felsineum: C. acetobutylicum* crosses is accounted for entirely by the common O factor. That between *C. acetobutylicum* and *C. roseum* is due to the O factor plus some admixture of H factors, the *C. acetobutylicum* containing a minor fraction of the H which is dominant in *C. roseum.* Thus, serological evidence confirmed in another instance the division of species proposed on morphological and physiological grounds.

Another butyric anaerobe, *Clostridium thermosaccharolyticum* McClung (185), was analyzed by the same technique (187). It also is homogeneous, with variation only in the quantity of H factor in certain strains. The extension of flagellar-somatic agglutination to this species is of particular interest, because, as a thermophile, the organism grows at a temperature which would destroy the H antigen of a mesophilic species. Yet relatively, the H factor of the thermophile is thermolabile and comparable to the same factor in other motile species (189).

THE APPLICATION OF THE PRECIPITIN AND COMPLEMENT FIXATION REACTIONS TO ANAEROBES

The precipitin reaction

The close analogy between the precipitin and agglutinin reactions is well-known. Although much less antigenic analysis has been done by precipitation, there is a good agreement of results obtained thus far by the two methods as applied to the spore-forming anaerobes. Detection of precipitins in the diagnosis of diseases has been attempted (91, 95, 133, 247, 270, 372), and precipitating sera for a number of the species of anaerobes have been successfully prepared (30, 40, 81, 108, 116, 351). Precipitins can often be demonstrated with antisera prepared for general antimicrobial use (302). Weinberg and Barotte (344) have claimed that precipitins and agglutinins act together in a serum to give stronger reactions ("synergism of antibodies") without loss of specificity.

Probably the most extensive work on precipitation for the typing of an anaerobic species has been done with *C. parabotulinum* by Gunnison and Schoenholz (116). A large collection of Type A

and B strains was tested in precipitating antisera produced with washed and heated cells, and good agreement with the grouping by agglutination was found. Later (108), antisera were produced with the bacterial cell extracts obtained by the freezing-thawing technique. Such extracts contained both protein and carbohydrate constituents, but they were not antigenic *in vivo;* they were, however, specifically precipitable by antisera produced against the intact organisms.

Comparatively little is known of the specific carbohydrate constituents of the anaerobes. Jimenez (155) reported simply that such a fraction had been isolated from *C. welchii*, and Meisel (218) reported similarly for a *Bacillus amylobacter*.

The complement fixation reaction

Much the same success can be claimed for typing by complement fixation as by agglutination and precipitation, but the method has been even less extensively used. It has had its most considerable trial with the botulinus organism (115, 306,) and the tetanus organism (83). Highly specific reactions with groupings closely following those revealed by agglutination have been found. Gunnison and Schoenholz (115) pointed out one advantage: namely, that certain so-called inagglutinable strains can be assigned to groups by complement fixation.

Mention might also be made of an incidental use of the complement fixation reaction for detecting the botulinus organism or its toxin in spoiled canned foods (160, 306). It is also possible to determine with a high degree of accuracy the toxin content of a culture (308).

This brief presentation of the precipitation and complement fixation phenomena of the anaerobes is not in proportion to the importance of these reactions. But it cannot be claimed that either of them has contributed much to the typing of anaerobes, except to confirm previously known groupings. Precipitin tests will doubtless contribute significantly, after the groundwork has been laid by studies of the specific soluble substances of these species.

A CRITIQUE OF SEROLOGY AS AN AID IN THE TAXONOMY OF ANAEROBES

Every bacteriologist must be troubled at times by problems of taxonomy. He may not himself be concerned with the technique and terminology of classification, but he would like to have *agreement* and *stability* as quickly as possible. To be told that bacteria are perhaps not classifiable is of no help. To be told that they can be classified only when more is known is of no present help. Nor is this quite true now, for there are groups of bacteria *within* which classification (in the common sense) has already been achieved. It is chiefly when he tries to comprehend the whole system of bacteria that he must conclude that taxonomy has failed. Yet he would probably still hold with Lehmann and Neumann (180) "that it is always necessary to strive after such a system."

The known best way to work toward a general classification is to build up one after another of the systems for special groups; and in that endeavor serological classification has its place. One can hardly expect to take an unknown organism, and knowing nothing of its morphology and physiology, to discover its identity by toxin neutralization. It may perhaps, produce no toxin. But one can, given a spore-forming anaerobe from the pulpy kidney, of a sheep, discover the toxin type. And having done so, one would have classified it intelligibly for others working with the *C. welchii* group. *Agreement* would be reached.

As to the *stability* of serological classification, it is possible to find arguments, both favorable and unfavorable. The major objections are that variation "changes" the antigenic reactions and that serology reveals a needlessly complex subdivision of organisms (which is perhaps another way of saying that antigenic variation has occurred). If the antigenic variation is so random that types are forever elusive, the objection is valid. But experience shows this not to be so. The very fact that a group of botulinus cultures, involving hundreds of isolations, can be sorted into five toxin types is significant. So too is the statement of Powell (251): "Uniformity in the results of agglutina-

tion and agglutinin absorption tests upon groups of single-cell cultures of the diphtheria bacillus, having common origins, indicates considerable stability in the agglutinative reactions of this organism. It has not been possible to split any parent culture on the basis of the agglutinative reaction of pure-line strains derived from it."

We do not imply that serological analyses have solved all problems of classification within groups. Certainly that cannot be claimed for any single method of serology. Agglutination of *C. welchii*, for example, has failed for reasons as yet unknown. We are aware also that there is no convenience in the considerable subdivision of *C. tetani* by its H antigen reactions; but we submit that the discovery of a common and species-specific O factor is useful. That the O factor of *C. parabotulinum* unites a part of the botulinus group is probably also significant. So also is the possible protective power of the "O vaccines" of *C. chauvoei*. So is the sharing of O factors between species in the butyric group. In short, it seems that *somatic* factors are most promising objects for further study. It is apparent also that group reactions require attention. The sharing of minor somatic elements among certain species (or among groups within a species) may show phylogenetic relationships useful to know. And conversely, removal of those group factors by pre-absorption of antisera may yield useful reagents for species analyses. One such case among the anaerobes has been indicated in discussion of *C. parabotulinum* and *C. sporogenes*.

Needless to say, all serological work requires great care. Improvement in purity and standardization of toxins and antitoxins, and greater use of cross-protection experiments for establishing the components of nearly related crude toxins, are the keys to further progress. In agglutination work greater emphasis must be placed on *complete* analyses with mirror absorptions. And finally, with precipitation reactions done upon isolated and if possible chemically defined fractions of protein and polysaccharide, it may be possible to reach that "Substantive Classification," which P. Bruce White has visioned (365).

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